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A Framework for Human Relevance Analysis of Information on Carcinogenic Modes of Action‡

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ABSTRACT: The human relevance framework (HRF) outlines a four-part process, beginning with data on the mode of action (MOA) in laboratory animals, for evaluating the human relevance of animal tumors. Drawing on U.S. EPA and IPCS proposals for animal MOA analysis, the HRF expands those analyses to include a systematic evaluation of comparability, or lack of comparability, between the postulated animal MOA and related information from human data sources. The HRF evolved through a series of case studies representing several different MOAs. HRF analyses produced divergent outcomes, some leading to complete risk assessment and others discontinuing the process, according to the data available from animal and human sources. Two case examples call for complete risk assessments. One is the default: When data are insufficient to confidently postulate a MOA for test animals, the animal tumor data are presumed to be relevant for risk assessment and a complete risk assessment is necessary. The other is the product of a data-based finding that the animal MOA is relevant to humans. *For the specific MOA and endpoint combinations studied for this article*, full risk assessments are necessary for potentially relevant MOAs involving cytotoxicity and cell proliferation in animals and humans (Case Study 6, chloroform) and formation of urinary-tract calculi (Case Study 7, melamine). In other circumstances, when data-based findings for the chemical and endpoint combination studied indicate that the tumor-related animal MOA is unlikely to have a human counterpart, there is little reason to continue the risk assessment *for that combination*. Similarly, when qualitative considerations identify MOAs specific to the test species or quantitative considerations indicate that the animal MOA is unlikely to occur in humans, such hazard findings are generally conclusive and further risk assessment is not necessary *for the endpoint–MOA combination under study*. Case examples include a tumor-related protein specific to test animals (Case Study 3, *d*-limonene), the tumor consequences of

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hormone suppression typical of laboratory animals but not humans (Case Study 4, atrazine), and chemical-related enhanced hormone clearance rates in animals relative to humans (Case Study 5, phenobarbital). The human relevance analysis is highly specific for the chemical–MOA–tissue–endpoint combination under analysis in any particular case: different tissues, different endpoints, or alternative MOAs for a given chemical may result in different human relevance findings. By providing a systematic approach to using MOA data, the HRF offers a new tool for the scientific community's overall effort to enhance the predictive power, reliability and transparency of cancer risk assessment.

KEYWORDS: risk assessment, carcinogenic mode of action, human relevance of animal carcinogens, acrylonitrile, atrazine, chloroform, ethylene oxide, *d*-limonene, melamine, phenobarbital.

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I. INTRODUCTION

This article has two parts and a short appendix. The first part provides background information on a new human relevance framework (HRF) for using mode of action (MOA) information to assess the relevance of animal tumors for human risk assessment. The second part presents seven case studies applying the HRF to MOAs postulated for several animal carcinogens. The chemicals differ as to available MOA data and related risk assessment requirements. The appendix highlights key elements in the risk assessment process.

II. A HUMAN RELEVANCE FRAMEWORK

A. Background

The National Research Council (NRC) "Red Book" paradigm for chemical risk assessment describes four fields of analysis, each with closely related but distinct information inputs and analytical results: hazard identification, assessment of dose-response relationships, exposure assessment for populations at risk, and risk characterization (NRC, 1983, 1994). Hazard identification is based on data and information from laboratory animal studies and, if available, human studies. In hazard identification and subsequent hazard characterization, the primary question is whether the effects observed in animal study populations might be expected in humans, a question that depends at least in part on extrapolating hazard and dose-response data from laboratory animals to humans. (Definitions and additional details on the NRC paradigm appear in the appendix.)

The MOA of the chemical under study is a fundamental aspect of these extrapolations (Vainio et al., 1992; IARC, 1992; NRC, 1994; NTP, 1998). To address this issue, the U.S. Environmental Protection Agency (EPA) and the International Programme on Chemical Safety (IPCS) have proposed

generally comparable guidance for evaluating animal MOA information to assess the relevance of animal tumors for human risk assessment (U.S. EPA, 1999, 2003; Sonich-Mullin et al., 2001). Both proposals focus on identifying "key events," generally described "as measurable effects that are critical to the induction of tumors, as hypothesized in the postulated mode of action." Similarly, both proposals offer specific guidance on developing and analyzing MOA information from *animal* studies, with considerably less guidance on applying this information to assess human relevance.

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The HRF expands the EPA and IPCS frameworks into a new four-part analysis (Table 1). The resulting system for human relevance analysis lies mainly in the hazard identification phase of the risk assessment. That is, where MOA information is available from both animals and humans, the HRF calls for a weight of evidence analysis and informed characterization of the available tumor data for potential human relevance. This characterization should be fully transparent as to data sources (both chemical-specific and generic), data gaps, assumptions about the applicability of generic data, and extrapolations within the MOA analysis. At the same time, the framework is nonprescriptive, offering a simple structure for making and articulating critical scientific judgments.

TABLE 1
The Human Relevance Framework

1. Is the weight of evidence sufficient to establish the MOA in animals?
   a. Postulated MOA
   b. Identification of key events
   c. Animal evidence
   d. Application of EPA/IPCS animal MOA guidance (Table 2)
2. Are key events in the animal MOA plausible in humans?
   a. Concordance analysis of animal and human responses
   b. Statement of confidence
3. Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?
   a. Concordance analysis of animal and human responses
   b. Statement of confidence
4. Statement of confidence; analysis; implications

The HRF adopts the customary presumption that animal tumors are relevant for human hazard or risk assessment (Interagency Regulatory Liaison Group [IRLG], 1979; Office of Science and Technology [OSTP], 1985; U.S. EPA, 1986, 1999, 2003). Similarly, the animal MOA is presumed to describe processes in humans as well as in animals. Although the presumption of relevance applies alike to DNA-reactive and non-DNA-reactive carcinogens, presumptive judgments of human relevance for non-DNA-reactive carcinogens often generate controversy and stimulate calls for MOA data to rebut the presumption as to individual chemicals. To augment guidance currently available on this contentious issue, this report focuses mainly on non-DNA-reactive carcinogens.

Employing an iterative approach, the Framework Subgroup combined case study methodology and current U.S. EPA and IPCS guidance proposals to assess the human relevance of animal carcinogens with well-studied postulated animal MOAs. The subgroup had several expectations: gaining experience in using the proposed guidance, identifying and isolating useful principles and approaches, and developing case studies that could serve as models for practitioners. As work progressed, completing the case studies required new approaches for using MOA analysis to determine whether or not related animal tumors provide data appropriate for human risk assessment. In this regard, three observations were pivotal points leading to the new four-part framework.

Limited Utility of Animal MOA Information

No matter how well-defined and fully analyzed, MOA information derived *solely* from animal studies does not permit definitive conclusions about human relevance or lack of relevance. Specifically, although an absence of human data permits an assumption of human relevance, conclusions about *lack of* human relevance depend in part on consideration of the potential applicability of the animal MOA to humans.

This observation is important because the literature on the MOA for chemical carcinogens in laboratory animals is expanding in several different ways. Information is available for increasingly more chemicals, and the new data are generally more reliable and more detailed. The result is enhanced understanding and confidence in methods for developing such MOA information. Moreover, completing an MOA relevance analysis generates new

questions and requires additional information, with the result that a well-defined animal MOA can stimulate research on the potential for human hazard and risk. Nonetheless, animal MOA data alone cannot answer the human relevance question.

Necessity for Human Information

To understand and describe the potential MOA in humans, information about humans that permits evaluating the applicability of the animal MOA is needed. Ideally, epidemiology studies would provide information such as biomarkers of exposure and of effect for use in MOA analysis. However, these studies are rarely designed to provide data to address MOA issues. Rather, pertinent information is available from other sources, generic and specific, with the most directly useful information coming from work involving the agent under study. Sources for such chemical-specific information include in vivo and in vitro studies and exposures at all levels of organization, ranging from population studies to cultured cells. Other sources include data from pathology specimens, diagnostic and other clinical tests, and from people with pertinent disease.

These human information sources may provide data derived from studies involving *other* chemicals (structurally or functionally related to the chemical under review) or *various* disease states. Such generic data are useful in addition to or in lieu of chemical-specific data, especially because data for specific chemicals may not be available or attainable. For example, MOA data on key events (or associated features) for other chemicals involved in the same disease process or the same or different chemicals in still other species may be used for this analysis. Information on comparative biochemistry, endocrinology, or physiology may be used. The essential question is biological plausibility in the sense of overall consistency between weight-of-evidence conclusions about the MOA in humans and established scientific information and principles. In this regard, consultation and collaboration to foster coordinated approaches to the conduct of epidemiology studies could enhance the database for MOA relevance analyses.

Utility of Comparative Analysis

Side-by-side comparisons of evidence relating to key events or associated features in animals and in humans promote information-based assessment of potential MOA comparability. Such MOA concordance analysis, which should not be confused with tumor site concordance, requires a systematic look at the key events identified for the animal MOA and determining if there are or may be comparable events in the human. As a minimum, a narrative description is necessary, and a "comparison" or "concordance" table may aid in developing and presenting the analysis.

Scope

Several limitations on the scope of the HRF and this report require attention. Most importantly, although there is no bright line between "mode of action" and "mechanism of action," this report deliberately focuses on the *mode* of action with only incidental references to *mechanism* of action. The MOA is a plausible hypothesis, supported by observations and experimental data, regarding events leading to a toxic endpoint. It describes chemical interactions with cellular components such that discerning the MOA involves identifying and measuring "key" cellular and biochemical events in the postulated pathway to carcinogenic or other changes. As a result, *mode* of action analysis may well incorporate data bearing on the *mechanism* of action. However, *mechanism* of action generally implies a detailed description and sufficient understanding of the molecular basis of an effect such as cancer to establish causation in molecular terms. Such mechanisms are seldom, if ever, fully known and this report does not attempt to describe them.

HRF analysis contributes to, but is not a substitute for, risk assessment. As noted earlier, the HRF analysis focuses mainly on the animal to human extrapolation in the hazard identification/characterization phase of risk assessment. The corresponding question in the dose-response analysis may depend in part on information used in or developed for the human relevance analysis, but additional considerations require applying the generally higher dose levels used in animal studies to estimate the hazard potential at the much lower exposure levels generally expected for human populations, especially for environmental chemicals. (This extrapolation does not apply, however, to pharmaceuticals or even to some components of food where animal and human exposure may be similar.) In this way, conclusions from the human relevance analysis are considered along with the dose-response and exposure analyses that are integral to quantifying and characterizing risk in line with the NRC risk assessment paradigm. The HRF analysis thus augments the risk assessment and contributes to confidence in conclusions about the carcinogenic potential for

humans and the likelihood and magnitude of human risk. Where HRF analysis precludes the need for continuing the risk assessment process (see below), the product is hazard characterization rather than risk characterization.

In addition to these limitations on the scope of this article, understanding the highly specific nature of human relevance analyses and conclusions is important. In the first instance, the MOA and related human relevance analysis for a particular chemical apply only for the endpoint and tissue type analyzed. That is, any particular chemical may, in fact, induce cancer through more than one MOA, be responsible for tumors in more than one tissue type, and affect endpoints other than cancer. As a result, any particular human relevance conclusion cannot be generalized to other MOAs, endpoints, or tissues without additional data and analysis. For example, the phenobarbital case in this report limits its analysis and conclusions to thyroid tissue, the tumor site analyzed for that case, and does not attempt to extend its conclusions to phenobarbitol-induced liver tumors, which would require a separate analysis. On the other hand, the perfluorooctanoic acid (PFOA) case in the peroxisome proliferation-activated receptor (PPAR)α agonist report analyzes MOA data for a chemical that induces tumors in three different tissues—in that case, liver, testis, and pancreas—with a separate analysis for each tissue. In addition, because chemicals may have several different MOAs, any conclusion regarding human relevance applies only to the MOA under study. For these reasons, human relevance conclusions cannot be treated as blanket statements extending to all elements of a toxicity profile. Rather, each human relevance conclusion is specific as to the MOA *and* tissue or cell type *and* endpoint analyzed.

B. HRF Components and Application to Case Studies

The HRF analysis moves from an initial focus on key events (and associated processes) in the animal MOA to a weight of evidence conclusion about the relevance to humans of the animal tumors under study. The analysis features three questions that guide the overall evaluation of human relevance and a final summary (Table 1).

To develop and test the HRF, the Framework Subgroup identified seven animal carcinogens for which substantial animal MOA data and information are available in the published literature. Chemicals were selected and analyzed to represent a specific MOA for the sole purpose of testing the framework rather than to summarize or re-evaluate the chemical. That is, chemicals were selected to represent fairly well-recognized MOAs and the data were not reanalyzed to confirm, modify or refute published animal MOA analyses.

Developing the case studies provided an iterative assessment of the framework and helped to uncover critical issues and problems that ultimately led to new approaches, principles, tools, and formats for MOA and relevance analyses. A discussion of the framework with a brief summary of how case studies contributed to its refinement follows.

1. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

Given a finding of tumors in animals, current U.S. EPA and IPCS guidance spells out topics for organizing and presenting the information at hand (Table 2) (U.S. EPA, 1999; Sonich-Mullin et al., 2001). This approach employs the criteria used by epidemiologists to assess causality. In adopting this approach, the IPCS emphasized that it "is not a checklist of criteria" but rather "an analytical approach to considering the weight of evidence for a mode of action (MOA) in a given situation" (Sonich-Mullin et al., 2001). As such, it is an important aid in identifying key events, uncertainties, and data gaps, and in determining whether the MOA is demonstrated.

The process begins with a statement of the proposed MOA and an enumeration of key events. The analysis continues with summaries of dose response and temporal relationships, along with analyses of the strength, consistency and specificity of key events, tumor responses, and biological plausibility and coherence. After considering other potential MOAs that may account for the tumors, the animal MOA analysis ends with an overall conclusion about the weight of evidence as to the MOA and the level of confidence in that decision. This MOA information is also essential for the evaluation of human relevance. The presentation identifies inconsistencies and data gaps to explain the weight of evidence and the level of confidence. It also provides a basis for identifying additional research needs.

The animal MOA analyses in the case studies in this report are organized in line with Table 2, which the Framework Subgroup found to be appropriate, helpful, and generally complete. Redundancies exist in the animal MOA analysis, but case study authors modified the presentations to minimize such

TABLE 2
Framework for Evaluation of an Animal MOA*

a. Postulated MOA
Brief description of the sequence of measured effects, starting with chemical administration, to cancer formation at a given site.
b. Key events
Clear description of each of the key events (measurable parameters) that are thought to underlie the MOA.
c. Dose-response relationships
Dose-response relationships identified for each key event, and comparisons presented of dose-response relationships among key events and with cancer.
d. Temporal association
Sequence of key events over time that lead to tumor formation.
e. Strength, consistency, and specificity of association of key events and tumor response
Complete assessment and presentation of the relationships among the key events, precursor lesions, and tumors. Portrayal of the consistency of observations across studies of different designs.
f. Biological plausibility and coherence
Determination of whether key events and the sequence of events are consistent with current biological thinking, both regarding carcinogenesis in general and for the specific chemical under review.
g. Other MOAs
Alternative MOAs that may be applicable for the chemical under review. Comparison of their likelihood vis-à-vis the proposed MOA.
h. Conclusion about the MOA
Overall indication of the level of confidence in the postulated MOA.
i. Uncertainties, inconsistencies, and data gaps
Identification of information deficiencies in the case; description of inconsistent findings in the data at large; evaluation of uncertainties; proposal of pointed research that could significantly inform the case.

*Adapted from U.S. EPA (1999), Sonich-Mullin et al. (2001).

repetition. The adequacy of the MOA data set is a fundamental component of this analysis. Data needs for determining how chemicals induce cancer in animals differ for different MOAs. In view of a myriad of different modes, developing criteria for determining what is required and whether enough information exists to establish a particular MOA is difficult. However, once a MOA has been well delineated for one chemical, data needs for verifying this mode for subsequent chemicals working through the same MOA will usually be significantly reduced.

Although this project focused on non-DNA-reactive carcinogens, a direct-acting alkylating agent, ethylene oxide (Case Study 1), is included to illustrate use of the framework for a representative genotoxic carcinogen. An International Life Sciences Institute Risk Science Institute (ILSI RSI) follow-on project is preparing a report on the value added of HRF analysis for genotoxic carcinogens.

However, if the MOA in animals is not well defined, questions of plausibility in humans and/or issues of quantitative differences between animals and humans are not useful for the evaluation of the carcinogenic potential. An example of an inadequate data set for delineating the animal MOA is provided in the case studies (Case Study 2, acrylonitrile). Although the available data suggest that oxidative damage may be responsible for acrylonitrile-related tumors in laboratory animals, the data do not adequately define the MOA. In addition, an alternative MOA involving DNA reactivity was explored, and it also was judged to be inadequate to account for the tumors. As a result, given only preliminary MOA information, the assessor must assume that animal tumors indicate a cancer potential for humans; a default risk assessment proceeds based on the potential hazard and risk to humans. This scenario applies to many chemicals for which preliminary information suggests, but falls short of establishing, a hypothesized MOA. In these cases, a full risk assessment for the endpoint is required.

2. Are Key Events in the Animal MOA Plausible in Humans?

This question represents a *qualitative* assessment of the relevance of the MOA to human cancer potential. Generally, the default assumes that animal tumors and the accompanying MOAs are relevant to humans. The animal MOA describes the

TABLE 3
Concordance of Animal and Human Key Events (and Associated Processes)

		Human and/or non-human primate	
Key event	Animal effect	Source of information	Effect
1.			
2.			
3.			
. . .			

pathway toward tumor development in terms of key events and associated features that describe or aid understanding tumor formation. Corresponding information on humans needs to be assembled and compared to that in animals. For example, one format (Table 3) aligns each key event (or other factor) in the postulated MOA with relevant information regarding both animal and human responses. Such a table can be useful and informative for assessing the strength of evidence supporting each event and facilitates the analysis of the qualitative relevance of the animal observations to humans.

Human information is typically of two types: One category deals with data on the specific chemical under evaluation (or chemically or functionally related agents), while the other involves generic information pertinent to the MOA but not necessarily derived from the agent under study. Both categories can contribute to the analysis; there is merit, however, in marking each key event as to whether the data arise from the chemical under test (or analogues) or from other sources. Where such data are available, the greater the degree of concordance between animals and humans for the assessed factors, the greater the confidence that the animal MOA is applicable to humans. In addition to the key events in the animal MOA, other useful information includes such factors as:

1. Cancer incidence at the anatomical site and cell type of interest, including age, sex, ethnic differences, and risk factors, including chemicals and other environmental agents.
2. Knowledge of the nature and function of the target site including development, structure (gross and histological), and control mechanisms at the physiological, cellular, and biochemical levels.
3. Human and animal disease states that provide insight concerning target organ regulation.
4. Human and animal responses to the chemical under review or analogs following short-, intermediate-, and long-term exposure, including target organs and effects.

It should be emphasized that data must be significant and convincing to deviate from the default and conclude that the MOA in animals is not relevant to humans. This requires substantial qualitative information indicating that humans are not expected to respond as do animals. If the data strongly support a species-specific MOA that is not relevant to humans, chemicals producing animal tumors by that MOA would not be expected to pose a cancer hazard to humans and no further risk assessment steps are necessary. Conversely, the default is retained and the animal MOA is presumed relevant for humans unless the animal MOA can confidently be rejected as not pertinent to human hazard potential.

To date, only a few MOAs for animal cancers have cleared the high hurdle to be generally accepted as not relevant to humans. Case studies are provided for $\alpha 2\mu$-globulin-associated male rat kidney tumors (*d*-limonene; Case Study 3) and inhibition of LH surge-related rat mammary tumors (atrazine; Case Study 4). These examples feature comprehensive data sets providing evidence of molecular or physiological characteristics that are unique to rats and that do not operate in humans. In the case of *d*-limonene, binding of a chemical metabolite to $\alpha 2\mu$-globulin in the kidney is the initial step in the carcinogenic process, resulting in renal tubule protein overload, cytotoxicity, reparative cell proliferation, and tumor development. There is a large body of data demonstrating that the presence of $\alpha 2\mu$-globulin is prerequisite to the development of this renal syndrome. Although this protein is present in the male rat, it is absent from female rats, mice (which show no evidence of renal tumorigenicity), and humans. No structurally related protein present in any other species, including humans, binds to a broad group of xenobiotics and elicits a renal syndrome in a manner similar to $\alpha 2\mu$-globulin. Thus, male rat kidney

tumors induced by this MOA are not considered applicable to human hazard.

The second case study where qualitative differences dictate the evaluation of human relevance of animal tumors is atrazine, which affects the hypothalamus of female Sprague-Dawley (SD) rats and leads to an inhibition of the luteinizing hormone (LH) surge during the estrous cycle. This sets into motion persistent secretion of estrogen and prolactin, leading to the development of mammary tumors. The hormonal changes and breast tumor response elicited by atrazine in SD rats are not seen in Fisher rats or CD1 mice. For this MOA and endpoint, physiological responses to sustained hormone secretion in humans are so different from the SD rat that, even if the human hypothalamus were affected in a manner similar to the SD rat, a totally different syndrome, namely, a hypoestrogenic state would be expected. Such an effect in humans would not lead to the induction of breast cancer.

3. Taking into Account Kinetic and Dynamic Factors, Is the Animal MOA Plausible in Humans?

If the animal MOA is judged to be *qualitatively* applicable to humans, an analysis of potential *quantitative* differences in sensitivity between animals and humans is necessary. Without such information, assessors usually assume that humans respond as do animals. Factors of interest include: differences in the biotransformation and effects of the chemical under investigation, that is, toxicokinetics (e.g., time course of chemical uptake, distribution, metabolism, excretion), and toxicodynamics (e.g., consequences of the interaction of the agent and tissues) that may increase or decrease susceptibility of humans relative to animals. Likewise, physiological, cellular, and biochemical differences between species regarding endogenous chemicals and control systems may require consideration.

As with the concordance analysis, a tabular display may provide a useful summary of the analysis of kinetic and dynamic factors relative to a MOA. Generally, the output of the analysis is a set of quantitative comparisons between animal and human responses, resulting in numerical differences in responsiveness. The remaining case studies (cases 5–7, respectively, phenobarbital, chloroform, and melamine) provide examples of quantitative differences that inform the evaluation in different ways. These cases illustrate a spectrum of quantitative differences and demonstrate how these differences can be applied to addressing human relevance. For example, with phenobarbital, multiple distinct differences exist that collectively suggest lack of relevance to humans. In contrast, the chloroform example presents a case in which processes leading to tumor formation are quite comparable across species, including humans. Melamine represents an intermediate case, where the differences between species impact the decision regarding the overall applicability of the animal MOA to human hazard.

Although hormonal axes are qualitatively similar across species, the disruption of the thyroid-pituitary status by phenobarbital (Case Study 5) presents a number of quantitative factors that determine relevance to humans. Phenobarbital enhances the hepatic clearance of thyroid hormone in rats by inducing the Phase II enzymes involved in thyroid hormone metabolism. This results in reduced circulating levels of thyroid hormone and triggers an increased output of thyroid-stimulating hormone (TSH). Sustained stimulation of the thyroid gland by TSH increases cell proliferation and leads to tumor formation. Rats are distinguished qualitatively from humans because they lack thyroxin-binding globulin, which transports thyroid hormone in the serum of humans. As a result, rats differ quantitatively from humans in that the half-life of thyroid hormone is much shorter, and TSH levels are higher than in humans. Consequently, humans are less likely to show such change in TSH levels. Like phenobarbital, none of the liver enzyme inducers is known to increase TSH in humans. In addition, although changes in hormone levels readily result in tumor development in rodents, especially the rat, this is not the experience in humans. These kinetic and dynamic factors argue persuasively that the MOA for phenobarbital related to the thyroid is not relevant to humans and no further cancer risk assessment is necessary.

The remaining case studies present examples in which animals and humans are qualitatively and quantitatively relevant regarding the animal MOA. In a case involving multiple tumors, separate analyses for the liver and kidney showed that cytotoxicity from chloroform (Case Study 6) depends on formation of a highly reactive metabolite, phosgene. This biotransformation step occurs in both rodents and humans, and exposed humans have shown toxicity in the same organs developing tumors in animals. Thus, the MOA in animals is relevant to humans. Toxicokinetic comparisons in both rats and humans show that the key to the MOA is the requirement for a dose sufficient to produce cytotoxicity and subsequent cellular regeneration.

In the case of urinary bladder stones resulting from exposure to melamine (Case Study 7), several qualitative and quantitative factors contribute to the overall MOA analysis. At high doses in animals, melamine precipitates in the urine to form calculi that accumulate in the urinary bladder. Stones in the bladder damage the urothelium, stimulating compensatory cell proliferation and tumors. In humans, calculi (formed from other substances) are more likely to be retained in the renal pelvis or ureters rather than in the bladder. Although bladder stones tend to be retained in the rodent bladder, they are more likely to be passed in the urine in humans or removed (surgically or by lithotripsy) because of urinary obstruction and pain. Overall, the relationship between urinary calculi and the development of bladder cancer in humans is not totally clear, but epidemiological data suggest a small relative cancer risk associated with the presence of urinary calculi in humans. Therefore, the framework analysis identifies this MOA as a potential human hazard requiring further risk assessment. For melamine, human exposure is the critical element that ultimately defines the potential for human risk. For both chemical cytotoxicity (chloroform) and urinary-tract calculi (melamine) MOAs, further risk assessment is necessary, including evaluation of the dose response and human exposure. In performing this risk assessment, it is presumed that knowledge regarding the MOA will be incorporated, providing scientific guidance for the evaluation.

An important consideration regarding the case studies concerns the issue of multiple tumor sites. For the chloroform case, liver and kidney tumors are generally considered to arise from the same MOA involving metabolic activation. In contrast, although phenobarbital is a widely recognized mouse liver carcinogen, the subgroup did not study the liver MOA and thus limited its human relevance conclusions to the thyroid. Overall, the MOA for each tumor type must be considered separately. Although the issue of multiple sites is a very important consideration in the risk assessment for a particular compound, a case example in which two tumor types arise from different MOAs was not included.

The decision analysis in Figure 1 summarizes the Framework Subgroup's human relevance conclusions for the nongenotoxic carcinogens analyzed for this report. (A future ILSI RSI report will comment on ethylene oxide and other genotoxic carcinogens.) As shown in the figure, when analyzed for human relevance, the MOAs for different chemicals fall out differently in terms of the critical question of human risk assessment. The weight of evidence analysis for each question as to species comparability determines placement on the figure. In general, when the human relevance analysis identifies a species-specific MOA, this characterization means that the animal MOA is unlikely in humans and there is little reason to continue risk assessment for that endpoint. On the other hand, comparable animal and human MOA findings indicate likely human relevance and call for a complete risk assessment.

4. Statement of Confidence, Analysis, and Implications

As appropriate, the statement of confidence should address such issues as the quality and quantity of data underlying the analysis, consistency of the analysis with the Framework set forth in Table 1, consistency of the database with the criteria set forth in Table 2, nature and extent of the concordance analysis, and likelihood of alternative modes of action.

C. General and Future Applicability of the HRF

The MOAs analyzed for human relevance for this report produced the specific results presented in Figure 1. Figure 2 is a general schematic illustrating the framework's capacity to accommodate other MOAs.

Although the HRF was developed using MOA data for non-DNA-reactive carcinogens, this approach may be useful in other areas. In particular, scientists are giving increasing attention to MOA analysis for health effects other than cancer. For example, MOA analyses are beginning to have a role in U.S. EPA assessments for noncancer effects (U.S. EPA, 2000, atrazine; U.S. EPA, 2002a, vinclozolin; U.S. EPA, 2002b, mesotrione). Similarly, Health Canada's Existing Substances Division has posted assessments for 2-BE (butoxyethanol) and carbon disulfide (Health Canada, 2002). Based on these examples, the concepts, analytical tools, and format guidance presented here for carcinogens merit consideration in MOA analyses for health effects other than cancer. Because several of the MOA case studies considered by the Framework Subgroup involve noncancer endpoints, the applicability of the proposed framework for endpoints other than cancer is apparent.

Similarly, the HRF may be useful for addressing questions regarding the relevance of animal

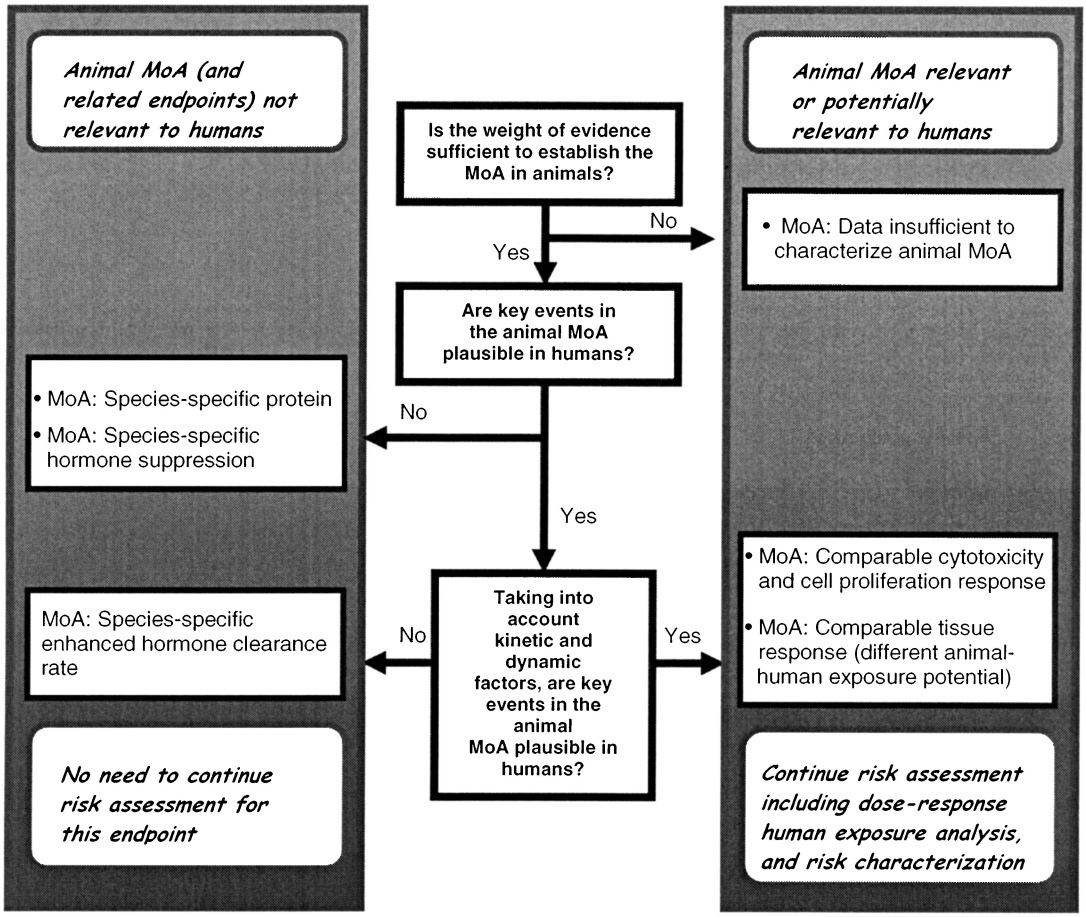

FIGURE 1. Schematic illustrating human relevance results for six case studies representing several different MOAs (and one example of data insufficient to characterize an animal MOA). The left side depicts data-based findings that the animal tumors are irrelevant because the MOA is unlikely to have a human counterpart due to a tumor-related protein specific to test animals (Case Study 3, *d*-limonene), the tumor consequences of hormones suppression typical of laboratory animals but not humans (Case Study 4, atrazine), and chemical-related enhanced hormone clearance rates in animals relative to humans (Case Study 5, phenobarbital). In cases where the animal tumor MOA is not expected in humans, the tumors are considered irrelevant and there is little reason to continue dose-response, exposure, and risk characterization for this endpoint. The cases involving relevant tumors on the right side portray one example of insufficient data (Case Study 2, acrylonitrile) and two examples that have human counterparts for the animal key events and associated processes. Full risk assessments are necessary for the insufficient data case, the MOA involving cytotoxicity and cell proliferation in animals and humans (Case Study 6, chloroform) and formation of urinary-tract calculi (Case Study 7, melamine). Risk estimates based on relevant tumors associated with different chemicals will vary with quantitative differences in exposure, toxicokinetics, and toxicodynamics.

tumors for assessing human risk at different life stages. A case-study approach would test applicability of the framework and identify appropriate refinements.

With these examples in mind, ILSI RSI is organizing a follow-on study to assess the value added of using the framework to evaluate the human relevance of other carcinogenic and noncarcinogenic endpoints.

D. Summary

Although current U.S. EPA and IPCS guidance offers excellent starting points for animal tumor MOA analysis (U.S. EPA, 1999; Sonich-Mullin et al., 2001), completing the case studies revealed several issues requiring special attention and new methods. In different ways, each case is instructive regarding information needed to more reliably

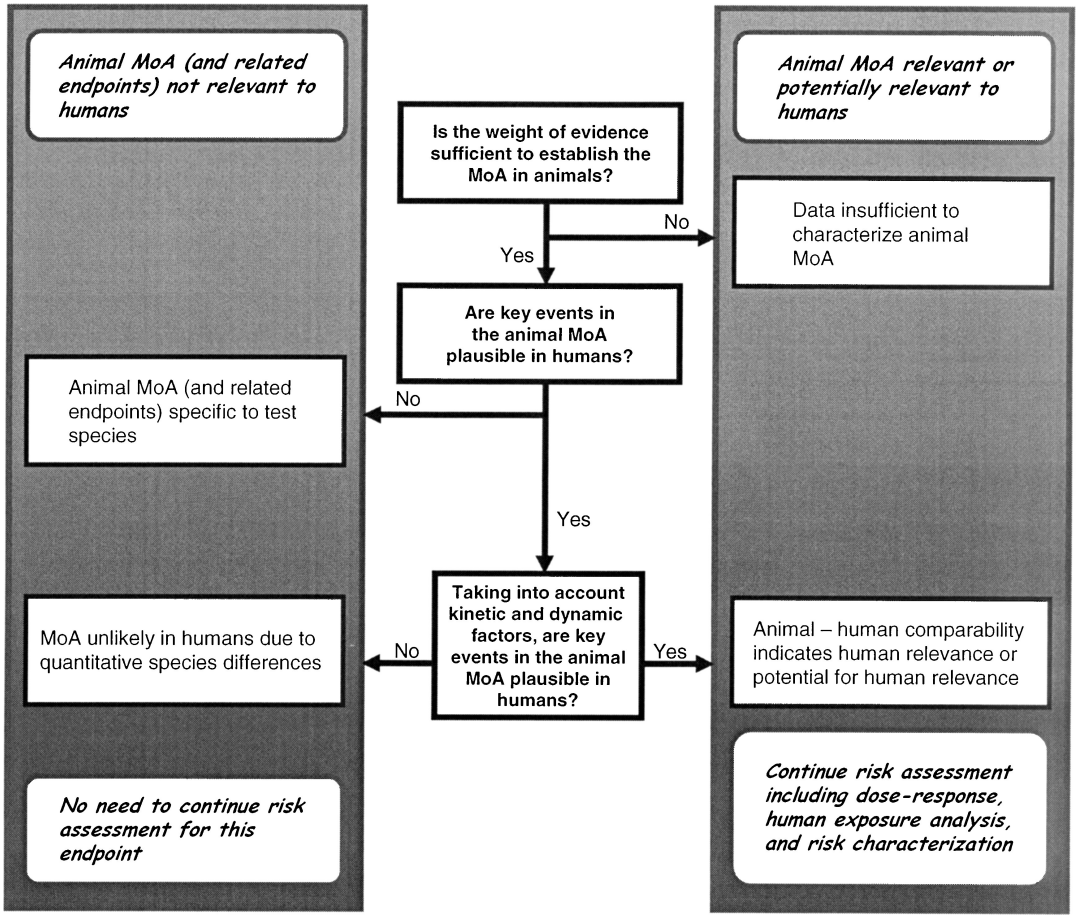

FIGURE 2. General schematic illustrating divergent outcomes for different MOAs analyzed in line with the four-part human relevance framework. The left side depicts data-based findings that animal tumors are irrelevant for human risk assessment because a tumor-related animal MOA is unlikely to have a human counterpart for the endpoint under study. When qualitative considerations identify MOAs specific to the test species or quantitative considerations indicate that the animal MOA is unlikely to occur in humans, such hazard findings are conclusive and there is little reason to continue risk assessment for that endpoint. The right side portrays two outcomes leading to complete risk assessments. One is the default: When data are insufficient to confidently characterize an MOA for test animals, the animal tumor data are presumed to be relevant to humans and a complete risk assessment is necessary. The other is the product of data-based findings that the animal MOA is relevant to humans: Risk assessment is required when the human relevance analysis shows qualitative or quantitative comparability between the test species and humans as to key events in the postulated mode of action (and related processes) for the endpoint under study.

assess the relevance, or lack of relevance, of animal tumors to humans. The cases make clear that characterizing a postulated MOA in laboratory animals is a necessary first step in a multipart analysis and that conclusions about the human relevance of animal tumors depend on comparing key events in the animal MOA with comparable information from human data sources. In addition, the cases demonstrate the importance of transparency—an explicit analysis of both qualitative and quantitative factors for evaluating the likelihood that the MOA postulated for animals can occur in humans.

The cases as a whole illustrate some of the issues and analyses that risk practitioners, scholars, and policymakers will encounter as the scientific and regulatory communities give increased attention to MOA information from animal and human studies

to evaluate the applicability of animal tumors to humans. As such, they also suggest new approaches to informed and consistent use of MOA information for health effects other than cancer and for different life stages.

III. CASE STUDIES

A. MOA: Direct Alkylation of DNA

Multiple Tumors Associated with Exposure to Ethylene Oxide (Case Study 1)

Alkylation of nucleophilic sites on nucleic acids by electrophiles is a well-studied and widely accepted mode of action of carcinogenesis (Pitot and Dragan, 2001). Ethylene oxide is a direct-acting alkylating agent that binds to nucleophilic sites on DNA and proteins. It is this characteristic that makes ethylene oxide an effective commercial sterilant/disinfectant and likely accounts for its carcinogenic activity.

The cancer database on ethylene oxide includes experimental animal studies by several routes of administration, as well as epidemiologic investigations of occupational exposures to workers using ethylene oxide as a sterilant, and during its production or other industrial use. There is a very rich set of data on the genotoxicity of ethylene oxide in many experimental systems spanning biological phyla from prokaryotes to humans. In addition, quantitative information, including physiologically based pharmacokinetic (PBPK) models, exists outlining the absorption, metabolism, and excretion in experimental animals and humans. Biomarkers of exposure and effect have been widely studied and described in the literature and are also incorporated into at least one PBPK model.

Ethylene oxide has been shown to induce sarcomas at the site of injection in female NMRI mice (Dunkelberg et al., 1981), although no skin tumors were observed in ICR/Ha Swiss female mice painted with ethylene oxide in acetone (~100 mg of a 10% solution) 3 times a week from 8 wk until death (Van Duuren et al., 1965). In inhalation studies, ethylene oxide increased the incidences of B6C3F1 mice with lung tumors and Harderian gland papillary cystadenomas in males and females, and malignant lymphomas, uterine adenocarcinomas, and mammary gland carcinomas in females (National Toxicology Program [NTP], 1987).

In two inhalation studies in Fischer rats, ethylene oxide induced brain gliomas and mononuclear cell leukemias, and peritoneal mesotheliomas in males. Similar tumor increases were seen in gliomas and in leukemias in the one study that employed female rats (Snellings et al., 1984; Garman et al., 1986; Lynch et al., 1984). In one study in which female Sprague-Dawley rats received ethylene oxide by gavage in vegetable oil, squamous-cell carcinomas of the forestomach were increased (Dunkelberg, 1982).

I. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

A. A Defined Mode of Action

The evidence for the involvement of DNA alkylation from ethylene oxide in causation of the tumors observed in experimental animals is indirect. Ethylene oxide produced tumors both at the sites of application (injection site in mice, lung in mice in inhalation study, forestomach of rats in gavage study) and at distant sites. There is evidence that exposure to ethylene oxide results in DNA adducts, mutations and/or other genetic effects at the site of contact as well as systemically. The International Agency for Research on Cancer (IARC, 1994) summarized in vivo inhalation studies in mice and rats, in which DNA adducts, primarily 7-hydroxyethyl guanine, were increased in the lung and liver as well as in other organs. Other adducts including O^6-(2-hydroxyethyl)guanine and N^3-(2-hydroxyethyl)adenine were found in some studies as well. Linear relationships were found between exposure concentrations of ethylene oxide and ethylene oxide adducts of hemoglobin and DNA.

Many studies have examined the formation of 7-hydroxyethyl guanine as to its contribution to the carcinogenic response of experimental animals to ethylene oxide. Walker et al. (1992) and Wu et al. (1999) exposed F344 rats and B6C3F$_1$ mice (via inhalation for 6 h/day, 5 days/wk for 4 wk) to concentrations of ethylene oxide similar to those used in previous carcinogenicity bioassays (Lynch et al. 1984; Snellings et al., 1984; Garman et al., 1985, 1986; NTP, 1987). Levels of 7-hydroxyethyl guanine were measured in several tissues (lung, spleen, brain, liver) and found to be generally higher in tissues of rats than mice. But within each species, similar levels of the adduct were measured in the respective tissues, and there was no clear distinction

between adduct levels in tissues that developed tumors versus those that did not.

Walker et al. (1997) showed that exposure to ethylene oxide induced mutations at the *hprt* locus in splenic T-lymphocytes in mice, and Sisk et al. (1997) demonstrated an increased frequency of *lacI* mutations in the lungs of transgenic mice exposed to ethylene oxide by inhalation, but not in the bone marrow or spleen. As with the DNA adduct studies, there was no clear relationship between the mutagenic response observed and the tissue-specific carcinogenicity of ethylene oxide.

In other studies, ethylene oxide caused an increase in sister chromatid exchanges (SCEs) in lymphocytes of rats, rabbits, and cynomologus monkeys, in bone marrow of mice and rats, and in the spleens of rats. Chromosomal aberrations were increased in the bone marrow of mice and rats exposed to ethylene oxide, and increases in bone-marrow micronuclei were seen in mice and rats (IARC, 1994). Although these studies demonstrate the range of genetic damage induced by ethylene oxide in experimental animals, a clear association with tissue-specific cancers has yet to be demonstrated for any of these endpoints.

Despite the absence of evidence of clear quantitative relationships between genetic damage in specific tissues and neoplasia, direct alkylation is considered causal for the carcinogenicity of ethylene oxide in experimental animals. Walker et al. (1992) have demonstrated repair of ethylene oxide-induced DNA adducts, and differences in the kinetics of formation and repair, coupled with other modifying factors, may make the expectation of a simple relationship between adduct load and tumors unrealistic. Nestmann et al. (1996) reported the discussions of an expert panel that considered the significance of DNA adducts in relation to carcinogenic risk. These discussions emphasized the lack of a simple relationship between adducts and tumors. They point out the importance of modifying factors such as the efficiency of conversion to mutations, and state that the presence of adducts does not necessarily represent a carcinogenic risk for a given tissue.

Exposure to ethylene oxide results in a wide spectrum of genetic damage as a consequence of DNA alkylation. The genotoxicity of ethylene oxide has been summarized by IARC (1994). Ethylene oxide has been found to induce genetic damage in prokaryotic, lower eukaryotic, higher plant, and in vitro and in vivo mammalian systems without additional metabolic activation systems, as well as in exposed workers. In bacterial systems, ethylene oxide induced mutations in *Salmonella, Escherichia coli*, and *Bacillus subtilis*. In lower eukaryotes, ethylene oxide induced gene mutations in *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Aspergillus nidulans, Neurospora crassa*, and *Drosophila melanogaster*. In higher plants, ethylene oxide induced chlorophyll and waxy mutations and chromosomal aberrations in barley, and mutations in rice and soybeans. In cultured mammalian cells, ethylene oxide induced SCEs in human fibroblasts, and SCEs and unscheduled DNA synthesis (UDS) in human lymphocytes. Gene mutations at the *hprt* locus were induced by ethylene oxide in Chinese hamster ovary cells, in lung V79 cells, and in mouse lymphoma cells. Evidence from some studies suggests that chromosomal deletions better account for the patterns of mutations than do point mutations (Sisk et al., 1997). Ethylene oxide-induced chromosomal aberrations were reported in transformed human amniotic cells, and in Chinese hamster V79 cells. Ethylene oxide induced transformation of C3H10 T1/2 and Syrian hamster embryo cells (IARC, 1994).

Dominant lethal mutations, heritable translocations (Generoso et al., 1990), chromosomal aberrations, DNA damage, and adduct formation (Sega et al., 1991) have been observed in sperm in a number of studies involving the exposure of rats and mice to ethylene oxide. Although there are no similar studies in humans, ethylene oxide should be considered a potential human germ cell mutagen.

B. Statement of Confidence: Supporting and Limiting Factors

Ethylene oxide has been found to be genotoxic in a wide variety of in vitro and in vivo systems. There is strong circumstantial evidence that ethylene oxide is genotoxic through its ability to directly alkylate nucleophiles including DNA. The confidence in this conclusion is high despite the lack of a clear quantitative relationship between DNA adduct levels and tumors in experimental animals.

II. Are Key Events in the Animal MOA Plausible in Humans?

There is considerable evidence to support the contention that ethylene oxide acts as a direct-acting alkylating agent in exposed humans. There is limited evidence from epidemiology studies that supports an association of exposure to ethylene oxide with several types of cancers that have also been

shown in experimental animals. In addition, numerous studies have monitored hemoglobin adducts and various genetic toxicity indices as biomarkers of exposure to ethylene oxide in occupational settings.

Lymphatic and hematopoietic cancers have been the most frequently reported cancers associated with ethylene oxide exposures in humans. Studies usually involved occupational exposures where ethylene oxide was used as a sterilant or ethylene oxide was produced or used to make other chemicals. Steenland et al. (1991) reported a cohort mortality study of 18,000 workers in 14 industrial plants in the United States that used ethylene oxide to sterilize medical devices or fumigate spices. The average follow-up time was 16 years and the average length of exposure was nearly 5 years. Although total hematopoietic tumors were not increased, standardized mortality ratios (SMRs) for lymphosarcoma–reticulosarcoma and kidney cancers were elevated but were not statistically significant. In a later report, Stayner et al. (1993), reanalyzed the Steenland et al. cohort applying industrial hygiene-based estimates of cumulative exposure. In this analysis, the SMR for all hematopoietic neoplasms was elevated in the highest exposure category, but a dose response was lacking. There was also a significant elevation of mortality from "lymphoid" cancers (non-Hodgkin's lymphoma and lymphocytic leukemia) in male workers with the highest cumulative exposures to ethylene oxide.

Shore et al. (1993) performed a meta-analysis of 10 cohort mortality studies published from 1979 to 1993 involving a total of 29,800 workers and 2540 reported deaths. The summary SMRs for leukemia and for non-Hodgkin's lymphoma were not significantly increased, although the authors recommended continued evaluation of these tumors because of limitations concerning exposure assessments and inadequate follow-up in some studies.

One limited study reported an excess of breast cancer in female sterilant workers (Norman et al., 1995). A follow-up of a cohort of Swedish sterilant workers first reported by Hagmar et al. (1991), was reported in Hagmar et al. (1995). Elevated but nonsignificant SMRs were found for lymphohematopoietic tumors and leukemia. Olsen et al. (1997) reported on workers in plants that produced ethylene oxide by the ethylene chlorohydrin process. This cohort mortality study also found elevated, but nonsignificant, SMRs for lymphopoietic and hematopoietic cancers.

As indicated earlier, numerous studies in the literature examine ethylene oxide-induced DNA adducts as well as other genotoxic endpoints, including chromosomal aberrations, strand breaks, mutations at the *hprt* gene locus, micronuclei, and sister chromatid exchange. Studies have also examined changes characterized as biomarkers of exposure, such as hemoglobin adducts. Preston (1999) has argued that many of the genotoxic endpoints measured in human occupational exposure studies are more appropriately considered biomarkers of exposure rather than predictors of subsequent adverse health effects. Nonetheless, detection of these endpoints in human tissue samples provides evidence of a direct alkylating activity following exposure to ethylene oxide.

Many of the earlier biomarker studies were summarized by the IARC (1994). There have been extensive studies relating the amounts of *N*-2(hydroxyethyl) adducts at histidine and *N*-terminal valine residues of hemoglobin to the air concentrations of ethylene oxide breathed by exposed workers. The binding of ethylene oxide to hemoglobin in washed red blood cells in vitro was studied by Segerback et al. (1990). The second-order rate constants for binding with valine and histidine did not differ between red blood cells of rats, mice and humans, although this was not the case for cysteine residues.

The IARC (1994) also reviewed 14 studies that reported an increase in the frequency of SCEs in the peripheral blood lymphocytes of ethylene oxide-exposed workers, and four studies that found equivocal or negative results. Ethylene oxide exposure was not associated with DNA strand breaks in one study, but was positive for DNA cross-links in another, and weakly positive for UDS in lymphocytes in a third. Three studies reported an increase in micronuclei in bone marrow, peripheral blood lymphocytes, and nasal cells of ethylene oxide-exposed workers, although four studies found no increase in micronuclei in lymphocytes, buccal cells, or nasal cells of exposed workers. Increases in chromosomal aberrations in blood lymphocytes of exposed workers were seen in eight studies, results were weakly positive in two studies, and negative in one.

Additional reports of evidence of chromosomal aberrations and micronuclei in ethylene oxide-exposed workers (Ribeiro et al., 1994), and increases in DNA damage as measured by alkaline elution of mononuclear blood cells of ethylene oxide-sterilization workers (Oesch et al., 1995) have been published since the IARC review. Schulte et al. (1995) reported an increase in SCE and micronuclei in lymphocytes in ethylene oxide-exposed female hospital workers, and Fuchs et al. (1994) reported increased DNA single strand breaks in

blood mononuclear cells in male and female hospital workers exposed to ethylene oxide. Other studies have failed to show increases in SCEs, *hprt* mutations, or micronuclei in lymphocytes of workers exposed to ethylene oxide in a manufacturing facility, or in studies of sterilization workers in 15 German hospitals (Popp et al., 1994; Tates et al., 1995).

In summary, although there are certain inconsistencies between some studies, the limited epidemiology findings suggestive of an association betweem ethylene oxide exposure and human cancers, combined with the considerable evidence for genotoxicity and adduct formation in human workers, provide strong evidence that the postulated mode of carcinogenic action of ethylene oxide can operate in humans.

III. Taking into Account Kinetic and Dynamic Factors, Is the Animal MOA Plausible in Humans?

Because the mode of action for ethylene oxide involves direct alkylation, quantitative differences in metabolic activation to a reactive intermediate are not an important consideration. However, the kinetics of tissue absorption, distribution, and elimination may affect the relative sensitivity of humans and experimental animals to the carcinogenic action of ethylene oxide.

Ethylene oxide is absorbed through human skin (Wester et al., 1997), and an estimated 75 to 80% of inhaled ethylene oxide is absorbed by the lungs and metabolized (Brugnone et al., 1986). Filser and Bolt (1984) determined that approximately 50% of ethylene oxide inhaled by Sprague-Dawley rats was absorbed and was systemically available. In rats a similar tissue:air partition coefficient was found for most organs, indicating a uniform distribution throughout the body (Krishnan et al., 1992), and a whole body:air concentration ratio of 1.88 was established for rats by Filser (1992). This is similar to the coefficient for venous blood:environmental air measured in exposed workers (Filser, 1992).

The half-life for elimination of ethylene oxide has been estimated to be 14 to 198 minutes in humans (IARC, 1994) and from 10 to 17 minutes in rats (Brown et al., 1996; Osterman-Golkar et al., 1983). Ethylene oxide is metabolized by hydrolysis and conjugation with glutathione, and excreted in urine as thioethers in humans, mice, and rats (IARC, 1994). Ethylene glycol has been found as a urinary metabolite of ethylene oxide in rats, mice and rabbits. As the concentration of inhaled ethylene oxide increases, the relative proportion metabolized in rats by hydrolysis to ethylene glycol increases and glutathione conjugation decreases (Koga et al., 1985, 1987). Fennell and Brown (2001) have developed a PBPK model that describes the exposure–tissue–dose relationships in rodents and humans, accounting for microsomal epoxide hydrolase mediated hydrolysis, and cytosolic glutathione *S*-transferase mediated conjugation. This model predicts the observed saturation of the glutathione pathway in mice inhaling ethylene oxide (Brown et al., 1998) at concentrations above 200 ppm.

The physiologically-based pharmacokinetic (PBPK) model for the dosimetry of inhaled ethylene oxide was first developed for rats, and included binding of ethylene oxide to hemoglobin and DNA in addition to tissue distribution, metabolic pathways (i.e., hydrolysis by epoxide hydrolase and conjugation by glutathione *S*-transferase), and depletion of hepatic and extrahepatic glutathione (Krishnan et al., 1992). The model was then refined and extended to mice and humans (Fennell and Brown, 2001). The models for rats, mice, and humans are qualitatively similar in their elements and provide for interspecies comparisons of internal ethylene oxide dose. The models are consistent with the conclusion that ethylene oxide is acting as a direct-acting alkylating agent in humans and rodents. Quantitative differences in biomarkers of exposure and effect are accounted for by differences in basic physiology between rodents and humans, rather than by factors suggesting a different mode of action.

Yong et al. (2001) found the level of hydroxyethyl hemoglobin adducts to be higher in ethylene oxide (EO)-exposed sterilizer operators who were null for the GSTT1 gene than in workers who had at least one copy of the gene. The GSTT1 status was not found to influence the level of SCEs in lymphocytes of exposed workers.

In summary, based on quantitative measurements of the comparative kinetics of absorption, distribution, metabolism and elimination, there does not appear to be any fundamental difference between experimental animals and humans that would suggest that the postulated mode of action does not occur in humans. This is supported by the biomarker data outlined in the previous section. At least one study has shown increased biomarkers of alkylation in exposed humans who had genetic deficiencies in a known detoxification pathway.

IV. Statement of Confidence; Analysis; Implications

There is adequate evidence from both experimental systems and human studies to test the strength and consistency of the hypothesis that direct alkylation accounts for the carcinogenicity of ethylene oxide. On a qualitative basis, there is also adequate biomarker and other genetic information to support the proposal that ethylene oxide is acting as a direct alkylating agent in humans. There is also adequate information to describe the quantitative aspects of the metabolism and direct alkylating capacity of ethylene oxide in humans and rodents. However, it must be pointed out that there is no specific information concerning exactly how much alkylation is necessary to increase the risk of cancer. Ethylene oxide is formed endogenously in mammals from the metabolism of ethylene, but Walker et al. (2000) have concluded that too little ethylene oxide arises from endogenous ethylene in rodents to produce a significant mutagenic response or a carcinogenic response under standard bioassay conditions.

There have been no credible proposed modes of action other than direct alkylation for the carcinogenicity of ethylene oxide.

The combined information on ethylene oxide from studies across phyla and extending into occupationally exposed workers provides a compelling body of evidence consistent with the involvement of direct alkylation in the carcinogenic action of ethylene oxide. The relationships between exposure, target tissue dose, and biomarkers of effect are understood quantitatively and qualitatively in experimental animals and in humans. At least one study has demonstrated increased biomarkers of alkylation in ethylene oxide-exposed workers who are genetically deficient in a detoxification pathway. Ethylene oxide has been found to induce adverse effects in a wide variety of assays for genetic damage. There is high confidence that direct alkylation is the mode of action for the carcinogenicity of ethylene oxide in humans and other mammalian organisms.

Utilizing the human relevance framework presented in the first section of this article, the answers to the questions regarding the weight of evidence for the animal MOA and the plausibility of the animal MOA in humans, together with the lack of modifying toxicokinetic and toxicodynamic factors, lead to the conclusion that the postulated MOA is applicable to humans, and that it is appropriate to perform a risk assessment to establish, quantitatively, risks to human health from exposure to ethylene oxide.

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B. MOA: Data Inadequate to Support Hypothesized Mode of Action

Brain Tumors Associated with Acrylonitrile Exposure (Case Study 2)

A range of tumors in rats—including those of the central nervous system (brain and/or spinal cord), ear canal, gastrointestinal tract, and mammary glands—has been consistently observed following exposure to acrylonitrile (ACN) by both ingestion and inhalation. Almost all adequate bioassays in rats have reported increases in the astrocytomas of the brain and spinal cord that are the focus of this case study.

In this case, the limited data available do not convincingly or consistently support the hypothesized mode of induction of the relevant tumor in animals (i.e., astrocytomas of the brain). Hence, the response to the first question in the human relevance framework—"Is the weight of evidence sufficient to establish the MOA in animals?"—is "no," and it is not possible to address the subsequent questions regarding the plausibility, qualitative or quantitative, of the mode of action humans. However, this does not preclude consideration of, for example, quantitative toxicokinetic variations between animals and humans in subsequent dose-response analyses.

I. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

A. Postulated Mode of Action/ Key Events

There is considerable evidence of the carcinogenicity of acrylonitrile, based primarily on early unpublished investigations in a single species (rats).[1] In the most sensitive bioassays, a range of

[1]Results of an NTP carcinogenesis bioassay indicate that acrylonitrile is also carcinogenic in mice exposed by gavage (NTP, 2001).

tumors (both benign and malignant) has been consistently observed following both ingestion and inhalation, including tumors of the central nervous system (brain and/or spinal cord), ear canal, gastrointestinal tract and mammary glands. In almost all adequate bioassays, increases in astrocytomas of the brain and spinal cord have been reported following inhalation (Quast et al., 1980a), ingestion in drinking water (Quast et al., 1980b; Bio/Dynamics Inc., 1980a, 1980b), and gavage (Bio/Dynamics Inc., 1980c). These tumors, for which dose-response trends are clear and which occur consistently at highest incidence across studies, are rarely observed spontaneously in experimental animals. Astrocytomas of the brain and spinal cord have sometimes been reported at nontoxic doses or concentrations and at periods as early as 7–12 months following the onset of exposure. In addition, tumors have also been observed in exposed offspring of a multigeneration reproductive study at 45 weeks (Litton Bionetics, Inc., 1980).

Acrylonitrile is metabolized primarily by two pathways: conjugation with glutathione to form *N*-acetyl-*S*-(2-cyanoethyl) cysteine, and oxidation by cytochrome P-4502E1 (Sumner et al., 1999) to form the remaining urinary metabolites (Fennell et al., 1991; Kedderis et al., 1993a). Oxidative metabolism of acrylonitrile leads to 2-cyanoethylene oxide, which is either conjugated with glutathione (Fennell and Summer, 1994; Kedderis et al., 1995) to form a series of metabolites including cyanide and thiocyanate or directly hydrolysed by epoxide hydrolase (Kim et al., 1993; Borak, 1992).

Available data[2] are consistent with conjugation with glutathione being the major detoxification pathway of acrylonitrile, while the oxidation of acrylonitrile to 2-cyanoethylene oxide can be viewed as an activation pathway, producing a greater proportion of the total metabolites in mice than in rats. Based on studies in which 2-cyanoethylene oxide has been administered, there is no indication of preferential uptake or retention in specific organs, including the brain (Kedderis et al., 1993b).

It has been hypothesized that acrylonitrile induces brain tumors through metabolically generated reactive oxygen species which cause damage to DNA. The hypothesized mode of action is not well defined, though oxidation to 2-cyanoethylene oxide by cytochrome P-4502E1 is a likely requisite initial step. Available data indicate that the events preceding hypothesized oxidative damage to DNA do not appear to involve significant disruption of antioxidant defenses of cytotoxic effects resulting in lipid peroxidation. Generated free radicals may be related to the release of cyanide in oxidative metabolism.

B. Evidence in Animals/Key Events

Key Events: Strength, Consistency, Specificity, Dose Response, Temporal Association, Biological Plausibility, and Coherence

The hypothesis that acrylonitrile induces brain tumors through metabolically generated reactive oxygen species that cause damage to DNA is based in part on a number of in vitro studies presented in abstract form, which indicate that free radicals ($\cdot OH$, $O_2\cdot$) and H_2O_2, generated perhaps in part by the release of cyanide, may be directly implicated in the oxidation of ACN and related DNA damage (El-zahaby et al., 1996, abstract; Mohamadin et al., 1996, abstract; Ahmed et al., 1996, abstract; Ahmed and Nouraldeen, 1996, abstract).

In more recent in vitro investigations, the results of which have also been presented in abstract form, Prow et al. (1997, abstract) reported that ACN inhibited gap junctional intercellular communication in a rat astrocyte cell line in a dose-dependent manner, possibly through an oxidative stress mechanism (Kamendulis et al., 1999a). Similarly, Zhang et al. (1998, abstract) concluded that oxidative stress contributed to morphological transformation in Syrian hamster embryo cells, assayed with and without an antioxidant (Zhang et al., 2000b). Jiang et al. (1998; abstract) reported oxidative damage (indicated by the presence of 8-hydroxy-2′-deoxyguanosine) at all concentrations tested in a rat astrocyte cell line and Zhang et al. (2000b) reported morphological transformation of Syrian hamster embryo (SHE) cells by 8-hydroxy-2′-deoxyguanosine.

In vivo data relevant to consideration of consistency of tumors with observations of key events are restricted to those from short term studies in which effects of ACN on levels of gonadotropin-stimulating hormone (GSH), reactive oxygen species, oxidative DNA damage, and measures of oxidative stress have been investigated. Studies of the association between the degree of metabolism to the putatively active metabolite, subsequent measures of reactive oxygen species or oxidative DNA damage and tumors are not available. In cancer bioassays, brain tumors occurred as early

[2]Including results of short-term toxicity studies in which the oxidative pathway has been induced prior to administration with acrylonitrile or antioxidants have been administered concomitantly with acrylonitrile.

as 7 to 12 weeks following exposure; however, information on progression of the lesions is sparse because very few of the early bioassays had interim kills, and none investigated reversibility.

Following exposure of Sprague-Dawley rats to 0 or 100 ppm ACN in drinking water for 2 weeks, levels of GSH and reactive oxygen species in brain and liver and levels of 8-hydroxy-2′-deoxyguanosine (indicative of oxidative DNA damage) and activation of NF-κB (a transcription factor strongly associated with oxidative stress) in several tissues were determined. GSH concentrations in brain were decreased (Jiang et al., 1997, abstract).

Whysner et al. (1998a) reported no effects on concentrations of GSH in the brain of male Sprague-Dawley rats exposed to 3, 30, or 300 ppm ACN in drinking water for 3 weeks, though there was a significant increase in 8-oxodeoxyguanosine levels in brain nuclear DNA at the two highest doses (NF-κB was also activated). In the liver, concentrations of nuclear DNA 8-oxodeoxyguanosine were also significantly increased at the two highest doses (Whysner et al., 1998a). In a bioassay with comparable dose levels, the incidence of brain and/or spinal cord tumors was significantly increased in male Sprague-Dawley rats exposed to 35 ppm (3.4 mg/kg body weight per day) ACN and higher for 2 years (Quast et al., 1980a).

In male F344 rats exposed for 21 days to 0, 1, 3, 10, 30, or 100 ppm ACN in drinking water, there were no significant differences between groups in 8-oxodeoxyguanosine levels in the brain (Whysner et al., 1997). (Note the contrast with reported results in Sprague-Dawley rats where there was an increase in 8-oxodeoxyguanosine in brain at 30 and 300 ppm for 3 weeks).

In male Sprague-Dawley rats exposed for up to 94 days to 0 or 100 ppm in drinking water, concentrations of 8-oxodeoxyguanosine in brain nuclear DNA were significantly increased after 3, 10, and 94 days of exposure (Whysner et al., 1998a). The endpoint for which changes were consistently observed in male Sprague-Dawley rats was the induction of oxidative DNA damage, including the accumulation of 8-oxodeoxyguanosine in the brain. The authors drew correlations between these results and the incidence of brain/spinal-cord tumors that had been reported in carcinogenicity bioassays in which male Sprague-Dawley rats were exposed to ACN via drinking water. In the two-year drinking water bioassay with male Sprague-Dawley rats (Quast et al., 1980a), the incidence of brain and/or spinal cord tumors was significantly increased at 100 ppm (8.5 mg/kg body weight per day).

Increased levels of 8-oxodeoxyguanosine occur only in the anterior portion of the brain, which contains rapidly dividing glial cells (Whysner et al., 1998b).

Increases in brain tumors have been dose related and observed at concentrations as low as 30 ppm in drinking water (equivalent to approximately 1 mg/kg body weight/day). However, in shorter term studies, in one strain but not in another where brain tumors were also observed, there were increases in 8-oxodeoxyguanosine in brain DNA (Whysner et al., 1998a, 1998b). Though there was a dose-response relationship in a 21-day study for increases in 8-oxodeoxyguanosine in the brain of Sprague-Dawley rats at concentrations similar to those at which astrocytomas were observed in this strain in carcinogenesis bioassays (Quast et al., 1980a), there was no such dose response for F344 rats, at doses at which brain tumors were observed in long term studies (Bio/Dynamics, 1980b).

In shorter term mechanism studies, therefore, exposure to acrylonitrile has been associated with the accumulation of 8-oxodeoxyguanine in the DNA isolated from brain tissue, presumably via the action of reactive oxygen species generated during its metabolism. However, the predicted greater sensitivity of Sprague-Dawley rats versus Fischer 344 rats based on 8-oxodeoxyguanosine levels in brain in shorter term studies is not borne out by carcinogenicity bioassays reported previously.

Moreover, acrylonitrile (and particularly the active metabolite 2-cyanoethylene oxide) has been shown to be mutagenic and produces DNA adducts in relevant assays (see "Alternate Modes of Action"). Therefore, direct interaction with DNA rather than indirect oxidative damage may be the critical key event.

Alternate Modes of Action

Potentially, acrylonitrile may induce brain tumors through direct interaction with DNA.

Acrylonitrile is weakly mutagenic in bacterial assays. However, the database on mutagenicity in mammalian cells in vitro and in vivo is inadequate as a basis for assessment. The few identified investigations comparing the relative potency of acrylonitrile to the epoxide metabolite are consistent with the oxidative pathway of metabolism being critical in genotoxicity.

Binding of 2-cyanoethylene oxide to nucleic acids has also been reported for in vitro studies at high concentrations (Hogy and Guengerich, 1986; Solomon and Segal, 1989; Solomon et al.,

1993; Yates et al., 1993, 1994). The formation of acrylonitrile–DNA adducts is increased substantially in the presence of metabolic activation. Under nonactivating conditions involving incubation of calf thymus DNA with either acrylonitrile or 2-cyanoethylene oxide in vitro, 2-cyanoethylene oxide alkylates DNA much more readily than acrylonitrile (Guengerich et al., 1981; Solomon et al., 1984, 1993). Incubation of DNA with 2-cyanoethylene oxide yields 7-(2-oxoethyl)-guanine (Guengerich et al., 1981; Hogy and Guengerich, 1986; Solomon and Segal, 1989; Solomon et al., 1993; Yates et al., 1993, 1994) as well as other adducts. Compared with studies with rat liver microsomes, little or no DNA alkylation by acrylonitrile was observed with rat brain microsomes (Guengerich et al., 1981). DNA alkylation in human liver microsomes was much less than that observed with rat microsomes (Guengerich et al., 1981); although there was no glutathione *S*-transferase activity in cytosol preparations from human liver exposed to acrylonitrile, there was some activity for 2-cyanoethylene oxide (Guengerich et al., 1981).

For in vivo studies in F344 rats administered 50 mg acrylonitrile/kg body weight intraperitoneally, 7-(2-oxoethyl)-guanine adducts were detected in liver (Hogy and Guengerich, 1986). Incorporation of acrylonitrile into hepatic RNA was observed following intraperitoneal administration to rats (Peter et al., 1983). However, no DNA adducts were detected in the brain, which is the primary target for acrylonitrile-induced tumorigenesis, in this or in a subsequent study in which F344 rats received 50 or 100 mg acrylonitrile/kg body weight by subcutaneous injection (Prokopczyk et al., 1988). In contrast, in three studies from one laboratory, exposure of SD rats to 46.5 mg [^{14}C]acrylonitrile/kg body weight (50 μCi/kg-bw) resulted in apparent binding of radioactivity to DNA from liver, stomach, brain (Farooqui and Ahmed, 1983), lung (Ahmed et al., 1992a) and testicles (Ahmed et al., 1992b). In each tissue, there was a rapid decrease in radioactivity of DNA samples collected up to 72 h following treatment.

It is not clear why acrylonitrile–DNA binding was detected in the brain in these studies and not by Hogy and Guengerich (1986) or Prokopczyk et al. (1988). The DNA isolation protocols and method for correction for contaminating protein in the DNA sample used by Hogy and Guengerich (1986) may have allowed a more stringent determination of DNA-bound material. Alternatively, the methods used to achieve greater DNA purity might have caused the loss of adducts or inhibited the recovery of adducted DNA. However, more likely, 7-oxoethylguanine and cyanoethyl adducts are of little consequence in inducing ACN-related brain tumors and investigation of the role of cyanohydroxyethylguanine and other adducts in inducing these tumors is warranted.

Therefore, while the role of mutagenesis and the primary mutagenic lesion induced by acrylonitrile in carcinogenesis are uncertain, acrylonitrile–DNA adducts [in particular, 7-(2-oxoethyl)-guanine] can be induced in vitro and in the liver in vivo, although at levels considerably less than those associated with, for example, ethylene oxide. However, when measures were taken to eliminate contamination of samples by adducted protein and unbound acrylonitrile, acrylonitrile–DNA adducts were not detected in the brain. This is in contrast to observations for ethylene oxide, which is also associated with gliomas of the brain. If the methods used to achieve greater DNA purity did not cause the loss of adducts or inhibit the recovery of adducted DNA, this suggests that acrylonitrile-induced DNA damage and mutagenicity may occur by a mechanism other than the formation of acrylonitrile–DNA adducts. Alternatively, they may be associated with an adduct (e.g., cyanohydroxyethyl adducts), another putatively critical adduct in brain that has never been investigated.

Moreover, several aspects of acrylonitrile-related tumor development are characteristic of tumors induced by compounds or metabolites which interact directly with DNA. Tumors are systemic and occur at multiple sites in both sexes following both inhalation and ingestion sometimes at nontoxic doses or concentrations and at periods as early as 7 to 12 months following onset of exposure. The ratio of benign to malignant tumors is small.

C. Conclusion: Assessment of Postulated Modes of Action in Animals and Statement of Confidence

Available data are insufficient to support a consensus view on a plausible mode of action for acrylonitrile-induced brain tumors other than through direct interaction with DNA. While there is some indication in ongoing studies, many reported in abstract form, that oxidative damage to DNA may play a role, available data are inadequate as a basis for delineation of a plausible sequence of events leading to cancer. For example, the origin of the oxidative damage is unclear. In vivo data on potential

key events for evaluating the weight of evidence for the hypothesized mode of action are limited to data from several strains of animals exposed for 21 days. Moreover, the pattern of results from these studies contrasts with those of the cancer bioassays, based on the hypothesized mode of action. The predicted greater sensitivity of Sprague-Dawley versus Fischer rats based on shorter term studies in which 8-oxodeoxyguanine levels in brain have been determined is not well reflected in the carcinogenesis bioassays.

With respect to a potential mode of induction of tumors involving direct interaction with DNA, there is evidence for the genotoxic potential of acrylonitrile in vitro, inadequate data in vivo, and insufficient data on acrylonitrile–DNA adducts in the brain, though such adducts can be induced in the liver in vivo.

Hence, while there is some indication in ongoing animal studies that oxidative damage to DNA may play a role in inducing these tumors, the origin of the oxidative damage is unclear. Moreover, the pattern of oxidative damage does not reflect well relative sensitivities of various strains to induction of tumors based on the hypothesized mode of action.

II. Are Key Events in the Animal MOA Plausible in Humans?

Section not relevant in this case, since weight of evidence for postulated mode of action for carcinogenesis in animals is inadequate.

III. Taking into Account Kinetic and Dynamic Factors, Is the Animal MOA Plausible in Humans?

Section not relevant in this case, since weight of evidence for postulated mode of action for carcinogenesis in animals is inadequate.

IV. Statement of Confidence; Analysis; Implications

In this case, the weight of evidence for the hypothesized mode of action (i.e., through metabolically generated reactive oxygen species that damage DNA) of the principal tumor type (i.e., astrocytomas of the brain) in animals is inadequate. In the context of the human relevance framework, then, the hypothesized mode of action is not considered further and available data are insufficient to support deviation from the default in dose-response analyses.

Although only one tumor type was considered in this case study, the weight of evidence for a hypothesized mode of action would need to be considered additionally and separately for each of the other animal tumors observed following exposure to acrylonitrile.

It should be noted, however, that while increases in astrocytomas of the brain and spinal cord have been reported in almost all adequate bioassays in rats exposed to acrylonitrile, increases in specific cancers have not been consistently observed in epidemiological studies of occupationally exposed populations. Although there was some evidence of excesses of lung cancer (Thiess et al., 1980), "all tumors" (Zhou and Wang, 1991), and colorectal cancer (Mastrangelo et al., 1993), primarily in early limited studies, consistent excesses of cancer have not been detected in four recent well-conducted and well-reported epidemiological studies in occupationally exposed populations (Benn and Osborne, 1998; Blair et al., 1998; Swaen et al., 1998; Wood et al., 1998). However, a nonsignificant excess of lung cancer was noted in the most highly exposed quintile in the statistically most powerful investigation (Blair et al., 1998). A large deficit in cancer in one cohort in comparison with national rates also suggests an underreporting of cause of death (Wood et al., 1998). The power to detect moderate excesses was also small for some sites (stomach, brain, breast, prostate, lymphatic/hematopoietic) because of small numbers of expected deaths. [For example, the upper 95% confidence limit on the SMRs for brain cancer in the only recent cohort study in which it was reported (Swaen et al., 1998) was 378, indicating that an almost 400% excess could not be excluded. The lower 95% confidence limit was 64.]

Unfortunately, the results of these epidemiological studies cannot contribute significantly to hazard characterization or dose-response analyses for acrylonitrile in part due to lack of a plausible mode of induction of tumors observed in the animal studies. Meaningful contribution of the observations is precluded by the relative paucity of data on exposure of workers in the relevant investigations and the wide range of confidence limits on the SMRs for rare tumors whose relevance cannot be precluded due to lack of understanding of the mode of induction of animal tumors. This observation has generic implications beyond this particular case study—that is, that results of negative epidemiological studies are rarely informative in the context of hazard characterization or dose-response analyses, particularly

where there is no plausible mode of induction of tumors, for which appropriate biomarkers of effect have been examined.

Data from animal studies consistently support that oxidation of acrylonitrile to 2-cyanoethylene oxide is likely a critical activation pathway in induction of tumors. Dose response might optimally be expressed, therefore, in terms of amounts or rates of formation of reactive metabolites produced per volume of tissue in the critical organ.

Based on studies in microsomes, the liver is the major site of formation in vivo of 2-cyanoethylene oxide in rats, mice and humans and studies with inhibitory antibodies in human hepatic microsomes indicate that the 2E1 isoform of cytochrome P-450 is primarily involved in epoxidation (Guengerich et al., 1991; Kedderis et al., 1993a).

Studies in subcellular hepatic fractions indicate that there is an active epoxide hydrolase pathway for 2-cyanoethylene oxide in humans, which is inactive, although inducible, in rodents (Kedderis and Batra, 1993). A physiologically based pharmacokinetic model has been developed and verified for the rat (Gargas et al., 1995; Kedderis et al., 1996), and work is under way to scale it to humans. In a recent, although reported in abstract form, study, Kedderis (1997) estimated in vivo activity of epoxide hydrolase in humans based on the ratio of epoxide hydrolase to P-450 activity in subcellular hepatic fractions multiplied by the P-450 activity In vivo. Human blood-to-air coefficients for acrylonitrile and 2-cyanoethylene oxide have also been recently determined, although reported in abstract form (Kedderis and Held, 1998). Research is in progress to determine partition coefficients for other human tissues.

M.E. Meek

C. MOA: Chemical-Induced Species- and Sex-Specific Protein ($\alpha 2\mu$-Globulin)

Male Rat Kidney Tumors Associated with Exposure to d-*Limonene (Case Study 3)*

Alpha 2μ-globulin is a protein that is synthesized in vivo in large quantities exclusively by male rats. The presence of this protein renders male rats uniquely sensitive to a chemically induced syndrome that is manifest acutely as the accumulation of $\alpha 2\mu$-globulin in renal proximal tubule cells, and which, with chronic exposure, progresses to renal tubular tumor formation.

d-Limonene, a monoterpene hydrocarbon that occurs naturally in citrus and other plant species, is a prototypical compound known to induce $\alpha 2\mu$-globulin nephropathy and renal tumors in male rats. *d*-Limonene is widely used as a flavor and fragrance in a variety of foods, beverages, and cosmetic products. It has also been shown to have benefit as a cancer chemotherapeutic agent, and continues to be evaluated in clinical trials for treatment of breast cancer and other tumors.

When dosed orally for 2 years to F344 rats, *d*-limonene caused a significant increase in renal tubular adenomas and carcinomas in male rats (75 and 150 mg/kg/day). No treatment related tumors were observed in female rats dosed up to 600 mg/kg/day or male and female mice dosed up to 1000 mg/kg/day (NTP, 1990). There is now a large and compelling body of data, comprising histopathological, biochemical, and molecular evidence, that establishes that the mode of action by which *d*-limonene is carcinogenic involves $\alpha 2\mu$-globulin. *d*-Limonene induces $\alpha 2\mu$-globulin nephropathy by binding exclusively to this protein and leading to renal protein overload. With chronic treatment, there is sustained protein overload, cell death, and compensatory renal cell proliferation, and these events represent a continuum that ultimately leads to renal tubular tumor formation. There is scientific evidence to support the conclusion that *d*-limonene-induced renal tubular tumors arising in male rats by a mechanism involving $\alpha 2\mu$-globulin, are unique to male rats and have no relevance to humans (Swenberg and Lehman-McKeeman, 1999).

d-Limonene represents a data-rich case study. There is considerable biochemical and molecular evidence to support the conclusion that a major qualitative difference, namely, the presence (or absence) of $\alpha 2\mu$-globulin, determines susceptibility to carcinogenicity.

I. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

The postulated mode of action by which *d*-limonene causes renal tubular tumors in male rats involves its ability to bind to $\alpha 2\mu$-globulin. This binding renders the protein more resistant to proteolytic degradation, which causes it to accumulate in renal phagolysosomes. The acute histopathological hallmark of this syndrome is the accumulation of protein (or hyaline) droplets in renal proximal tubule cells. The syndrome is often referred to as $\alpha 2\mu$-globulin or hyaline droplet nephropathy

denoting the morphological changes observed with protein-engorged lysosomes. With chronic treatment, the renal protein overload causes renal cell injury and compensatory cell proliferation. Sustained cell proliferation leads to a low but significant incidence of renal tubular tumors (Swenberg and Lehman-McKeeman, 1999).

There are two key events involved in the process by which *d*-limonene is carcinogenic in male rats. First, a metabolite of *d*-limonene, *d*- limonene-1,2-epoxide, binds reversibly, but specifically to $\alpha 2\mu$-globulin. The dissociation constant for this binding is approximately 1×10^{-7} M (Lehman-McKeeman and Caudill, 1992a). *d*-Limonene is a member of a diverse group of compounds that produce this unique male rat-specific renal toxicity and for all compounds, the ability to bind to $\alpha 2\mu$-globulin appears to be the rate-limiting step in the development of the nephropathy and tumors (Swenberg and Lehman-McKeeman, 1999).

It is important to note that *d*-limonene is not DNA reactive. It shows no evidence of mutagenicity or clastogenicicty in standard tests (NTP, 1990). Furthermore, *d*-limonene 1,2-epoxide is an unusually stable intermediate, and it has also produced negative results in a battery of mutagenicity tests (Basler et al., 1989).

The second key event is sustained renal cell proliferation resulting from continued *d*-limonene treatment. With chronic renal cellular protein overload, there is continued renal cell necrosis and compensatory cell proliferation. Renal cell proliferation occurs almost exclusively in the P_2 segment of the proximal tubule, the major site of protein reabsorption in the tubule (Swenberg and Lehman-McKeeman, 1999; Borghoff et al., 1990).

There is very strong evidence supporting the essential role that $\alpha 2\mu$-globulin plays in the development of the renal syndrome and carcinogenic response. Alpha 2μ-globulin is an unusual protein that is synthesized in the liver by adult male rats where it is under complex hormonal control. Female rats possess the entire compliment of hepatic $\alpha 2\mu$-globulin genes, but estrogen is a very effective repressor of its expression (Roy et al., 1975). In the absence of $\alpha 2\mu$-globulin, female rats show no evidence of renal toxicity following *d*-limonene treatment, even at much higher dosage levels. Similarly, the male NCI Black Reiter (NBR) rat, an unusual strain that does not synthesize $\alpha 2\mu$-globulin, is resistant to renal toxicity (Dietrich and Swenberg, 1991a). Mice synthesize a protein that is more than 90% homologous with $\alpha 2\mu$-globulin, but this protein does not bind *d*-limonene metabolites and no renal toxicity is observed. However, transgenic mice engineered to express $\alpha 2\mu$-globulin develop protein droplet nephropathy after *d*-limonene treatment (Lehman-McKeeman and Caudill, 1992b, 1994). In all cases, the metabolism and disposition of *d*-limonene is similar, supporting the conclusion that the presence of $\alpha 2\mu$-globulin is prerequisite to the development of renal toxicity (Dietrich and Swenberg, 1991b; Lehman-McKeeman and Caudill, 1994).

The lack of DNA reactivity of *d*-limonene and the epoxide intermediate supports the concept of a nongenotoxic mode of action. Although *d*-limonene-induced renal cell proliferation is observed in male rats, no similar changes are noted in female rats. Furthermore, *d*-limonene did not increase renal cell proliferation in male NBR rats, demonstrating that increased renal cell proliferation is entirely dependent on the presence of $\alpha 2\mu$-globulin (Dietrich and Swenberg, 1991b).

An initiation–promotion study, conducted with *N*-nitroso-ethylhydroxyethylamine (EHEN) and *d*-limonene, compared the histopathology, cell proliferation and tumor response in F344 and NBR rats. In this study, *d*-limonene treatment increased renal cell proliferation, atypical hyperplasia and renal adenoma formation. In contrast, no change in cell proliferation or renal tumor incidence was observed in the NBR rats. These data demonstrate that *d*-limonene acts as a renal tumor promoter and that this response requires $\alpha 2\mu$-globulin (Dietrich and Swenberg, 1991b).

There is also good concordance in male rats for the dose-response relationships that define *d*-limonene-induced $\alpha 2\mu$-globulin nephropathy, renal cell toxicity, and cell proliferation. The dosages shown to be carcinogenic produce a significant response in all three parameters. Furthermore, chronic studies indicate that continued treatment is necessary to sustain renal cell proliferation (Swenberg and Lehman-McKeeman, 1999).

One possible uncertainty in the mechanism of $\alpha 2\mu$-globulin nephropathy is the discontinuity between the acute nephropathy and renal tumor outcome. Although not shown with *d*-limonene, a few compounds have produced the acute syndrome of $\alpha 2\mu$-globulin nephropathy, but have not produced renal tubular tumors. In this regard, differences in binding affinity to $\alpha 2\mu$-globulin and the overall severity of the ensuing nephropathy affect the level of sustained cell proliferation. It appears that there is a critical level of regenerative cell proliferation that must be reached for renal tumors to develop (Swenberg and Lehman-McKeeman, 1999).

Although the role of $\alpha 2\mu$-globulin in this renal syndrome is clear, a few alternative hypotheses have been developed. One alternate hypothesis suggests that *d*-limonene is directly nephrotoxic and that binding to $\alpha 2\mu$-globulin concentrates the nephrotoxic compound in the kidney. However, studies with *d*-limonene have shown no evidence of renal toxicity in female rats when dosed subchronically with up to 10% in the diet (Elson et al., 1988) or up to 2400 mg/kg by gavage (NTP, 1990). In contrast, dosages of 75 or 150 mg/kg were shown to be nephrotoxic and carcinogenic in male rats. Although there are no pharmacokinetic data available to evaluate renal tissue burdens, it is reasonable to conclude that the renal concentrations of *d*-limonene achieved at the very high dosages tolerated by female rats are at least equal to the renal concentrations achieved in male rats at much lower dosages.

The overall biological plausibility and coherence of the postulated mode of action for *d*-limonene-induced renal tubular tumor formation is very strong. Studies across species demonstrate that the response is unique to male rats, and studies with NBR rats and transgenic mice prove that $\alpha 2\mu$-globulin is required for the development of nephrotoxicity, cell proliferation, or renal tumor formation.

II. Are Key Events in the Animal MOA Plausible in Humans?

For the mode of action underlying the development of $\alpha 2\mu$-globulin nephropathy in male rats to apply to humans, two important criteria must be met. First, metabolism of the inducing chemical to the species known to bind to $\alpha 2\mu$-globulin would have to occur. Second, and more importantly, $\alpha 2\mu$-globulin or a homologue of this protein must be present in humans.

Studies on the fate and toxicity of *d*-limonene in humans have shown that the monoterpene is well tolerated (Igimi et al., 1976). In patients treated with *d*-limonene (0.5–12 g/m_2/day for 21 days) the peak plasma concentration of *d*-limonene ranged from 10.8 to 20.5 μM, and a predominant metabolite detected in plasma was *d*-limonene 1,2-diol (10–20 μM), the hydrolytic product of the corresponding epoxide (Vigushin et al., 1998). Hence, humans are capable of metabolizing *d*-limonene to the metabolite known to bind to $\alpha 2\mu$-globulin in rats.

Given that the presence of $\alpha 2\mu$-globulin is necessary for the development of renal tubular tumors in rats, considerable experimental effort has been directed at determining whether there is a human homologue of $\alpha 2\mu$-globulin. Alpha 2μ-globulin belongs to a family of superfamily proteins that share an unusual tertiary structural motif that forms a hydrophobic binding pocket. These proteins, referred to as the $\alpha 2\mu$-globulin superfamily, bind and transport hydrophobic ligands. Proteins such as retinol-binding protein, $\alpha 1$-acid glycoprotein, and apolipoprotein D are members of this family (Flower et al., 1993), which is present in all mammals. Therefore, in vivo studies showing no evidence of renal (or other) toxicity in female rats, male and female mice, dogs, or monkeys support the position that structurally similar proteins do not contribute to a similar syndrome in any species, including humans (Lehman-McKeeman, 1997). As a direct determination of whether superfamily proteins could function in a manner similar to $\alpha 2\mu$-globulin, their ability to form *d*-limonene-1, 2-epoxide has been evaluated. Although binding to $\alpha 2\mu$-globulin can be readily demonstrated in vitro, the epoxide did not bind to any other superfamily protein (Lehman-McKeeman and Caudill, 1992a). Additionally, there is in vivo evidence that there are no proteins in human kidney that could function in a manner analogous to $\alpha 2\mu$-globulin. Specifically, after an exhaustive analysis, no protein that could bind to *d*-limonene 1,2-epoxide was isolated from human kidney (Borghoff and Lagarde, 1993).

A comparison of the key events noted in rats relative to humans is summarized in Table 4.

For the key events noted in Table 4, the strength of evidence of data obtained from laboratory animals and humans is extremely convincing. In addition to the lack of any human homologue identified by binding characteristics, there is also molecular evidence that $\alpha 2\mu$-globulin is an unusual and uncommon protein. Specifically, the x-ray crystal structure of $\alpha 2\mu$-globulin has been derived, and these results have revealed that the xenobiotic binding pocket of $\alpha 2\mu$-globulin is unique among the members of the superfamily. In particular, the binding pocket is nearly round and very open, allowing for the binding of a diverse group of compounds. In contrast, other superfamily proteins have much flatter and restricted shapes, which limit ligand binding (Swenberg and Lehman-McKeeman, 1999).

III. Taking into Account Kinetic and Dynamic Factors, Is the Animal MOA Plausible in Humans?

Section not relevant in this case because key events in the animal MOA are not plausible in humans.

TABLE 4
Comparison of Key Events in Rats Relative to Humans

Key event	Evidence in animals	Evidence in humans
Metabolism to *d*-limonene 1,2-epoxide	Observed in rats and mice	*d*-Limonene 1,2-diol detected in plasma, indicating formation
Binding to $\alpha 2\mu$-globulin	Present only in male rats	Not present; no other human protein can substitute
Renal syndrome of hyaline droplets and cell proliferation	Observed only in male rats when $\alpha 2\mu$-globulin present	No evidence

IV. Statement of Confidence; Decision Analysis

d-Limonene induces renal tubular tumors exclusively in male rats, operating by a mode of action that occurs only in male rats. The lack of toxicity in female rats, NBR rats, or male or female mice, along with evidence of toxicity in an $\alpha 2\mu$-globulin transgenic mouse model, proves that synthesis of $\alpha 2\mu$-globulin is necessary for the development of this renal syndrome. The critical question for addressing human relevance of $\alpha 2\mu$-globulin nephropathy and male rat-specific renal tumor formation is whether any protein in humans can function as a surrogate for $\alpha 2\mu$-globulin. A compelling body of evidence indicates that no such surrogate exists. Accordingly, the mode of action by which *d*-limonene causes renal tumors in male rats does not operate in humans.

There is a wealth of experimental data establishing the mode of action by which *d*-limonene causes renal cancer in male rats. After an exhaustive review of the *d*-limonene data set and data from other chemicals that induce this syndrome, criteria for establishing whether a chemical causes renal tumors through a mode of action involving $\alpha 2\mu$-globulin have been developed. These criteria include (Swenberg and Lehman-McKeeman, 1999):

- Lack of DNA reactivity.
- Male-rat-specific nephropathy and renal tumorigenicity.
- Histological evidence consistent with protein droplet accumulation.
- Identification of $\alpha 2\mu$-globulin in renal protein droplets.
- Reversible binding of chemical or metabolite to $\alpha 2\mu$-globulin.
- Induction of sustained cell proliferation in the renal cortex.
- Similar dose-response profiles for histological endpoints and renal tumors.

V. Implications

These criteria demonstrate the need for convincing experimental evidence of the key events of tumor development in rodents, which then guide the determination of human data needed to establish relevance of the tumor findings to humans. Furthermore, *d*-limonene represents a data-rich example of the extent of evidence needed to establish a mode of action for the first time. When qualitative differences dictate tumor susceptibility, as in this case, it is essential that the key events be well-defined and tested in laboratory animals and humans. In this specific example, the identification of $\alpha 2\mu$-globulin was achieved with a proteomic analysis of male rat kidney proteins, and mutant and transgenic animal models were invaluable tools to demonstrate the requirement for $\alpha 2\mu$-globulin in the acute and chronic syndrome. After establishing that protein binding was a prerequisite to the development of hyaline droplets, the ability to directly evaluate this key event by determining the binding properties of structurally similar proteins in other species, especially humans, was essential to establishing the lack of human relevance of this animal syndrome and carcinogenic response.

Lois Lehman-McKeeman

D. MOA: Suppression of Luteinizing Hormone

Mammary Tumors Associated with Atrazine Exposure in the Female SD Rat (Case Study 4)

One way that a chemical can increase the incidence of cancer is by amplifying or suppressing normal, background cellular processes. Insofar as that cellular process operates across a wide range of

species, including humans, then that chemical and mode of action have human health relevance. On the other hand, where cellular processes in laboratory animals are restricted to a specific species, strain, or sex, then the human health relevance is considerably different. This case study illustrates a species-specific mode of action by showing how atrazine exacerbates and accelerates reproductive aging in the Sprague-Dawley (SD) rat, causing an earlier onset and higher incidence of mammary tumors in female SD rats.

Atrazine has been manufactured and used as a broad-spectrum herbicide for approximately 40 years in the United States and throughout the world and is one of the most highly used herbicides in the United States today. The carcinogenic potential of atrazine has been investigated in a number of studies on different strains of rats and mice (Stevens, 1994). The tumorigenic response observed in rodent bioassays is limited to one sex of one strain, the female SD rat.

Several studies have shown that high doses of atrazine caused an increased incidence and/or earlier appearance of spontaneously occurring mammary tumors in the female SD rat. There has been no consistent association between atrazine treatment and the incidence of any other tumor type in bioassays with female rats. In a serial sacrifice study (Thakur, 1991a), pituitary tumors occurred earlier in the 400-ppm atrazine group, but not in the 70-ppm group, compared to the untreated control group. There was no effect of treatment on pituitary tumor incidences in the 400-ppm atrazine group after 24 months.

Atrazine had no carcinogenic effect in the male Sprague-Dawley rat, the male and female Fischer 344 rat, or the male and female mouse in three tested strains. Furthermore, the untreated Fischer 344 female had a very low spontaneous incidence of mammary tumors compared to a high spontaneous incidence found in the concurrent study conducted with the SD rat, a factor that is significant in describing the strain-specific nature of the atrazine-related mammary tumors.

The genotoxic potential of atrazine has been examined in a broad array of tests. Atrazine is considered not to be genotoxic; therefore, the mode of action is not via direct action on DNA.

Work done on luteinizing hormone suppression as atrazine's mode of action in causing mammary tumors exclusively in female SD rats is extensive and fits well with the stepwise human relevance framework presented in the introduction to these case studies.

I. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

A. Postulated Mode of Action

Given atrazine's lack of genotoxic potential and lack of Tier I or Tier II estrogenic action, other causes of mammary tumors in SD rats were investigated. In evaluating the effects of atrazine on mammary tumor development in the female SD rat, an experimental basis has been established for a cascade of endocrine-related changes beginning with luteinizing hormone (LH) surge suppression, followed by estrous-cycle disruption, leading to an earlier appearance and/or a higher incidence of fibroadenomas and adenocarcinomas. This pattern of endocrinologic aging has been extensively described for the female SD rat. High doses of atrazine accelerate the normal reproductive aging process in this strain of rat.

Furthermore, the response observed in the female SD rat is unique to this strain of rat, since neither the Fisher 344 (Thakur, 1991b, 1992) rat nor three strains of mice (Sumner, 1981; Hazelette and Green, 1987; Innes et al., 1969) have demonstrated any tumorigenic effect in lifetime bioassays. In addition, low doses of atrazine, even in the highly sensitive SD rat, have no effect on LH, estrous-cycle disruption, or mammary tumor development. The lack of a tumorigenic response at low doses of atrazine ($\sim$3.5 mg/kg/day) is due to the operation of a physiologically based threshold. High doses of atrazine suppress LH release such that there is an insufficient titer of LH in the blood to trigger ovulation. When ovulation fails, follicles within the ovum continue to produce estrogen for another day until another ovulatory LH surge occurs. Repetitive failure of ovulation creates a state of persistent estrus resulting in the hyperstimulation of the mammary gland by estrogen and prolactin.

In chronic bioassays on natural and synthetic estrogens, it has been established that prolonged stimulation of the mammary gland with estrogen leads to development of adenocarcinomas (Cutts and Noble, 1964). In contrast, high-level stimulation of the mammary gland with prolactin has been shown to be linked to the development of fibroadenoma as a result of ductal enlargement, lobuloalveolar development, the development of secretory activity, and the formation of milk cysts (galactoceles) (McConnell, 1989, 1995; Gullino, 1975; Banerjee et al., 1994; Meites, 1972). A schematic

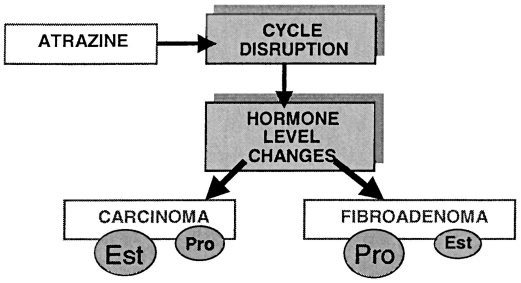

FIGURE 3. Postulated mode of action for atrazine.

of the postulated mode of action is shown in Figure 3.

B. Key Events

The key events include suppression of the LH surge, thereby prolonging estrous and attendant peak estrogen and prolactin levels, which leads to mammary tumors in the female SD rat. The conceptual framework that has emerged is that atrazine interacts with molecular targets (membranes, receptors, neurotransmitter systems) within the hypothalamus. This leads to a suppression of the intermittent secretion of gonadotropin-releasing hormone (GnRH) during critical phases of the normal rodent estrous cycle, which is comprised of 1 to 2 days in diestrus (D), 1 day in proestrus (P), and 1 day in estrus (E), as shown in Figure 4.

The consequence of this primary event is that luteinizing hormone released from the pituitary is of insufficient amplitude or duration to trigger the ovulation of developing follicles in the ovary. The failure to ovulate in the female Sprague-Dawley rat is a key event that leads to prolonged exposure to endogenous estrogen and/or prolactin for each additional day that the animal spends in a state of estrus or diestrus. Ovulation in the Sprague-Dawley rat has a biologic threshold. Either the animal ovulates or it does not. Either the atrazine dose is sufficient to suppress LH to block ovulation or it is not.

The net consequence of ovulatory failure is that the mammary gland is hyperstimulated by estrogen arising from the follicle and prolactin produced by the pituitary. Over a sufficiently long period of time, this hyperstimulation translates into a proliferative response in the mammary gland characterized by the development of adenocarcinoma (high estrogen, moderate prolactin levels) or fibroadenoma (high prolactin with a background of estrogen).

The cascade of events triggered by high doses of atrazine administered to female SD rats is qualitatively similar to that observed in spontaneously aging Sprague-Dawley females. The major difference is that in atrazine-treated female SD rats, the pituitary/hypothalamic failure occurs earlier in life. The Fischer 344 rat is refractory to these changes because the reproductive aging process is dissimilar from that occurring in the Sprague-Dawley rat.

1. Specificity and consistency: Effect of atrazine on LH and gonadotropin-releasing hormone (GnRH)

Studies indicated (Cooper et al., 1995, 1996) that 2- to 3-week pretreatment of Long-Evans rats with high doses of atrazine (75 to 300 mg/kg/day) suppressed both serum LH and prolactin surges in ovariectomized animal (Long-Evans and the Sprague-Dawley rats are outbred strains derived from the Wistar rat). Daily intraperitoneal

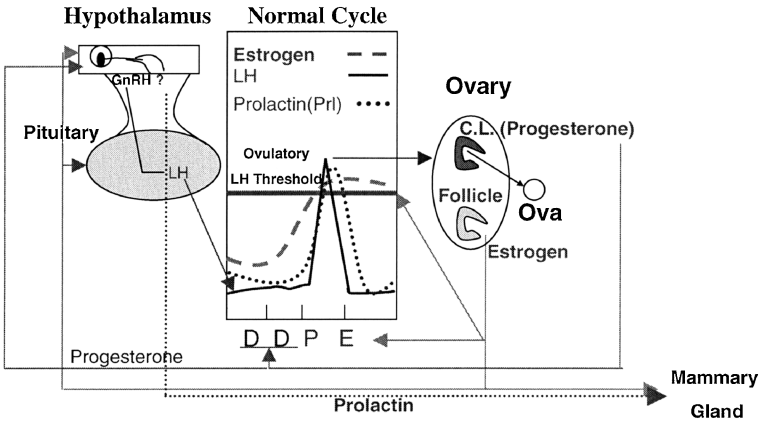

FIGURE 4. Schematic of the normal estrous cycle and ovulatory luteinizing hormone threshold.

administration of atrazine to ovariectomized rats at a dose level of 200 mg/kg (but not 50 mg/kg/day) for 3 days markedly inhibited episodic pulses of LH and significantly suppressed the serum LH level relative to controls (Tyrey et al., 1996). These results have been confirmed in short duration studies (Morseth, 1996a; Simpkins et al., 1998).

A 6-month feeding study indicated that LH levels were significantly suppressed only at feeding levels (400 ppm) where a mammary tumor response was observed in chronic bioassays (Morseth, 1996b). No significant effect of atrazine treatment on the LH surge was observed at feeding levels $\leq$50 ppm. This feeding level was not associated with an earlier appearance and/or an increased incidence of mammary tumors in a recent chronic bioassay on atrazine.

The ability of atrazine to inhibit the ovulatory surge of LH indicates a site of action at either the level of the hypothalamus or pituitary. Action within the hypothalamus could deprive the pituitary of the GnRH signal necessary for LH release, whereas effects within the pituitary might diminish the secretory response to that signal or processes involved in the biosynthesis of LH. Normal concentrations of LH and prolactin were found in the pituitary, suggesting that the hormones had not received the appropriate release signals. High doses of atrazine led to a reduction in hypothalamic norepinephrine, suggesting a hypothalamic site of action (Cooper et al., 1996).

The retention of pituitary responsiveness to GnRH stimulation after atrazine treatment (300 mg/kg/day $\times$ 3 days) was confirmed by the demonstration that a normal serum LH surge was reinstated in atrazine-treated animals if GnRH (50 ng/kg) was administered.

2. Specificity and consistency: Effect of atrazine on the estrous cycle

Animals in the 6-month study conducted to evaluate the effect of atrazine treatment on the LH surge had daily vaginal smears collected in alternating 2-week blocks of time. Based on cytological characteristics, the smears were classified for each day as being proestrus (P), estrus (E), or diestrus (D).

High-dose atrazine animals began to develop abnormal estrous cycles after approximately 10 weeks of treatment as indicated by an increased proportion of time spent in estrus. Control animals also displayed an increased number of abnormal days in estrus after 10 weeks but not to the same extent as the high-dose atrazine animals. There was no effect of treatment on the estrous cycle at feeding levels of $\leq$50 ppm.

As indicated previously, these events result in an earlier onset and higher incidence of mammary tumors in SD rats, but not in Fischer 344 rats. The control Fischer 344 female rat does not display abnormal estrous cycles until late in life and it does not develop a high incidence of mammary tumors. A high dose of atrazine in Fisher 344 rats had no effect on either the estrous cycle or mammary tumor incidence. The results reinforce the perspective that female Sprague-Dawley rats display an earlier disruption of the estrous cycle than control animals and develop mammary tumors earlier. Disrupted estrous cycles precede the appearance of mammary tumor development in both the control and treated groups; the effect of high doses of atrazine is to make both of these events occur earlier.

3. Strength: Differential prediction of mammary adenocarcinomas and fibroadenomas

Predicting when an animal was likely to develop a mammary fibroadenoma was significantly increased if information on mammary secretory activity, the presence of galactocele or pituitary tumors, the percent of time spent in diestrus or abnormal diestrus, and the percent time spent in estrus was known (Sielken et al., 1999). None of these variables was important for predicting mammary adenocarcinomas except for data on the percent time spent in estrus or abnormal estrus. These results, which are derived from mathematical modeling, are consistent with expectations based on an understanding of factors that impact adenocarcinoma and fibroadenoma development in the female Sprague-Dawley rat.

C. Alternative Modes of Action

1. Genotoxic potential

Atrazine was evaluated for its genotoxicity potential in more than three dozen assays including in vitro and in vivo gene mutation, chromosomal aberration, and other tests (Brusick, 1994). The overall weight of evidence from these tests indicates that atrazine is not genotoxic. Six out of 38 mutagenicity studies evaluated were positive. Other studies using the same type of test system

were negative. Furthermore, atrazine did not induce a tumorigenic response in ovariectomized female Sprague-Dawley rats (Morseth, 1998). Genotoxic carcinogens like dimethylbenz[a]anthracene (DMBA) (Yoshida et al., 1982; Clinton et al., 1995) and *N*-methyl-*N*-nitrosourea (MNU) (Gullino et al., 1975), on the other hand, are capable of inducing mammary tumors in ovariectomized rats.

2. Estrogenic potential

The estrogenic potential of atrazine has been thoroughly examined by using the proposed EDSTAC (Endocrine Disruptor Screening and Testing Advisory Committee) screens and tests. The experimental evidence supports that atrazine has no intrinsic estrogenic potential, based on the lack of response in either the proposed EDSTAC screens (uterine weight, estrogen receptor binding, transvected mammalian and yeast cell models) (U.S. EPA, 1998). The Tier II test (two-generation reproduction study) was also negative. Although there was a possible suggestion that atrazine may possess some weak antiestrogenic activity (Tennant et al., 1994; Tran et al., 1996), this finding was not replicated (Connor et al., 1996; Graumann et al., 1999).

D. Conclusions

The postulated mode of action is supported by data showing that atrazine-treated SD rats maintain constant estrous. The strength, consistency, and specificity of the available mode of action information for SD females is further confirmed by data showing that Fischer 344 (F344) rats, which have a different reproductive senescence, do not form atrazine-related mammary tumors. Alternative hypotheses have been ruled out, such as genotoxicity or estrogenicity, further showing the strength and specificity of the proposed mode of action.

II. Are Key Events in the Animal MOA Plausible in Humans?

The mode of action for the formation of mammary tumors in SD rats depends on the rodent-specific nature of the reproductive cycle and reproductive senescence. Because the cycle and senescence processes are fundamentally different in SD rats versus humans and nonhuman primates (Ordog et al., 1998; Schiff and Wilson, 1978), humans appear unlikely to have the potential to have LH suppression result in sustained estrous and hyperstimulation of breast tissue by estrogen and prolactin.

Basic physiological differences between SD female rats and humans underlie the question of human relevance. The defining event in atrazine-related mammary tumors in SD female rats is suppression of the LH surge (Tyrey et al., 1996; Morseth, 1996b; Simpkins et al., 1998; Eldridge et al., 1998; Aschheim, 1976; Meites et al., 1978). This feature, along with the nature of reproductive aging in the SD rat, illustrates why atrazine is unlikely to cause mammary tumors in humans. In the SD female rat, atrazine's effect of LH surge suppression and consequent sustained levels of estrogen and prolactin hastens the aging process, leading to an earlier onset and higher incidence of mammary tumors. This would not occur in humans, whose reproductive cycling, aging, and hormone levels are substantially different.

This case offers a particularly rich database for comparing key events in the mode of action. A full concordance or comparison analysis is facilitated by the availability of data on atrazine in animals and humans and by the extensive literature on reproductive endocrinology in both animals and humans. The concordance analysis for atrazine, summarized in Table 5, examines key events in the mode of action in terms of biological plausibility, strength, consistency, specificity, dose response, and the temporal association of tumor response with the key events.

A. Plausibility: Comparison of the reproductive cycles in rodents and humans

In the rodent, the estrous cycle is short and the preovulatory LH surge is brief, timed by the light cycle and dependent on the brain. The brain plays a deterministic role in the LH surge in rodents. Every afternoon during a critical period, a brain signal for LH secretion occurs that is driven by the increased activity of norepinephrine neurons (Sawyer, 1975; Simpkins et al., 1979a, 1979b; Wise et al., 1997). As such, selective blockage of this increased activity of norepinephrine neurons during this brief period blocks the preovulatory LH surge (Simpkins et al., 1985).

The human menstrual cycle is long, exhibits a protracted preovulatory LH surge and ends with

TABLE 5
Comparison of Key Events in Rats and Humans

Key event	Evidence in animals (rats)		Evidence in humans
	Sprague-Dawley	F-344	
Decreased GnRH pulses	Yes	No	Unknown/possible
Suppression of LH surge	Yes	No	Unknown/possible
Change in cyclicity	Yes	No	No/not relevant
Prolonged increase in estrogen/prolactin	Yes	No	No

menses due to the death of the corpus luteum and the resulting decline in estrogens and progestins. The driving force for the preovulatory LH surge in women is ovarian estrogen secretion (Ordog et al., 1998). The role of brain regulation of GnRH is that the preovulatory LH surge is permissive in women and other primates (Ordog et al., 1998; Krey et al., 1975; Goodman and Knobil, 1981; Pohl and Knobil, 1982). Indeed, the entire menstrual cycle can be recapitulated in rhesus monkeys in which the source of GnRH has been destroyed, by exogenous administration of pulses of GnRH (Ordog et al., 1998; Krey et al., 1975; Goodman and Knobil, 1981; Pohl and Knobil, 1982). In contrast to the observations in rodents, inhibitors of norepinephrine neurotransmission do not affect the preovulatory LH surge in women or other primates (Weiss et al., 1977; Knobil, 1974).

B. Plausibility: Comparison of reproductive aging in rodents and humans

Reproductive aging in rodents and women also is distinctively different. In female SD rats, reproductive senescence is a result of a breakdown of the brain regulation of the LH surge, while the ovaries are functional very late into life (Aschheim, 1976; Meites et al., 1978). The decline in reproductive function is primarily a result of the inability of brain norepinephrine neurons to transmit the estrogen signal to GnRH neurons (Simpkins et al., 1979a, 1979b; Wise et al., 1997; Meites et al., 1978). The inability to stimulate a preovulatory LH surge results in the maintenance of ovarian follicles and the persistent secretion of estrogens. Sequentially, the increased secretion of estrogens causes a persistent state of hyperprolactinemia (Sarkar et al., 1982). Thus in the SD rat, reproductive senescence is characterized by persistent hyperestrogenemia and hyperprolactinemia with low levels of LH and follicle-stimulating hormone (FSH).

In women, reproductive aging is characterized by exhaustion of ovarian follicles and the resulting menopause (Schiff and Wilson, 1978). During menopause, the ability to induce a preovulatory LH surge is normal, but estrogens, the driving force for the cycle, are absent. Postmenopausal estrogens and prolactin are very low, but LH and FSH secretion in women remains high.

In addition, epidemiologic studies indicate that there is no known association between atrazine and any cancer (Loosli, 1995; Sathiakumar and Delzell, 1997; Neuberger, 1996). Three types of studies (retrospective case-control studies, follow-up, studies, and ecologic or correlational studies) have assessed possible relationships between atrazine or other triazine herbicides and cancer in humans. Each study has shortcomings that limit conclusions. For example, limitations of the ecological studies include: (1) atrazine and other exposures were not measured at the individual subject level, but rather at the geographic regional level; (2) exposures were measured virtually concurrent with cancer occurrence; (3) factors such as duration of exposure and time since first exposure could not be analyzed; and (4) control for confounding was not adequate. Nonetheless, the weight of evidence from these studies indicates that atrazine is unlikely to cause cancer in humans.

Furthermore, an ongoing worker health survey in an atrazine manufacturing plant shows no relationship of atrazine to any cancer type. Prostate cancer incidence was elevated in this study, but is attributed to high rates of prostate-specific antigen (PSA) testing as part of the plant's medical program compared to the reference population.

III. Taking into Account Kinetic and Dynamic Factors, Is the Animal MOA Plausible in Humans?

Section not relevant in this case because the weight of evidence for the postulated mode of action for carcinogenesis in animals is not relevant in humans.

IV. Statement of Confidence; Decision Analysis

The postulated mode of action is supported by data showing that atrazine-treated SD rats maintain constant estrus. The strength, consistency, and specificity of the available mode of action information for SD females is further confirmed by data showing that Fischer 344 rats, which have a different reproductive senescence, do not form atrazine-related mammary tumors. A concordance analysis comparing key events in the animal mode of action with related reproductive processes in the human female shows distinct differences. Alternative hypotheses have been ruled out, such as genotoxicity or estrogenicity, further showing the strength and specificity of the proposed mode of action. Because luteinizing hormone as the mode of action for tumor formation appears to be specific to the female SD rat and does not appear to have a counterpart in the human female, atrazine-related mammary tumors formed by this mode of action in the SD rat are qualitatively not relevant for human risk assessment.

In summary, a detailed, comprehensive, and experimentally supported description of the mode of action underlying the mammary tumor response observed in SD rats at high doses of atrazine has been presented. This is supported by a rich research literature on endocrinological aging in various rodent species, and an extensive histological description of the effects of various hormonally active drugs including oral contraceptives.

- The mammary tumor response in atrazine-treated female SD rats is mediated by a nongenotoxic, threshold-based mechanism leading to LH suppression, failed ovulation, and estrous-cycle disruption.
- The mammary tumor response in atrazine-treated female SD rats occurs as a result of estrous-cycle disruption, which leads to an endocrine environment (prolonged exposure to endogenous estrogen associated with extended estrus) favorable to mammary tumor formation.
- A likely site of action of atrazine is the hypothalamus, since the pituitary LH response to exogenously administered gonadotropin-releasing hormone (GnRH) was restored after high-dose atrazine treatment if GnRH was administered.
- By inhibiting the LH surge and subsequent ovulation, atrazine exacerbates a condition to which the SD rat is normally predisposed and that is, in fact, the normal cause of reproductive senescence in this strain.
- The available data establish a causal relationship between atrazine exposure, an altered hormonal environment (resulting from estrous-cycle disruption), and the occurrence of mammary tumors in the SD rat.

V. Implications

Applying this logic in the human relevance framework analysis allows answers to the proposed questions. For HRF Question 1, "Is the weight of evidence sufficient to establish the MOA in animals?," the answer is clearly "Yes." For HRF Question 2, "Are key events in the animal MOA plausible in humans?," the answer is "No," ending the relevance analysis for mammary tumors in Sprague-Dawley rats formed through suppression of luteinizing hormone and lessening the need to continue the risk assessment for these tumors.

Timothy Pastoor

E. MOA: Increased Hepatic Clearance of Thyroxin and Thyroid Carcinogenesis

Thyroid Tumors Associated with Exposure to Phenobarbital (Case Study 5)

There are various non-DNA-reactive compounds that cause thyroid tumors in rats in which circulating thyroid hormone levels are decreased as a result of increased hepatic metabolism and clearance. These compounds induce hepatic glucuronidation of thyroid hormone and increase biliary excretion of the conjugated hormones. As a result of the hypothyroid state, thyroid-stimulating hormone (TSH) levels increase and cause sustained

thyroid follicular-cell hyperplasia, leading to tumor formation.

Phenobarbital (PB), a widely used barbiturate, is a classical inducer of hepatic xenobiotic-metabolizing enzymes. In rats, PB treatment induces cytochrome P-450 (CYP) enzymes along with Phase II conjugating enzymes, particularly UDP-glucuronosyltransferases (UDPGTs) and glutathione *S*-transferases (Waxman and Azaroff, 1992). At dosages associated with high levels of enzyme induction, PB is representative of the class of xenobiotics that alters thyroid hormone homeostasis and promotes thyroid tumor development in rats. PB has been studied in initiation–promotion models where tumor response has been shown to be directly proportional to the increased plasma TSH concentrations (McClain and Rice, 1999). The induction of thyroid tumors is observed in rats, whereas thyroid tumors are not seen in mice. It is widely recognized that PB is also a rodent liver carcinogen. However, for the present case study, only the mechanism and human relevance of thyroid carcinogenesis are discussed.

The case study presented here features both qualitative and quantitative differences between rodents and humans. The basic physiology and feedback mechanisms of the hypothalamic–pituitary–thyroid axis are qualitatively similar across species. However, quantitative differences are the major determinants used to assess human relevance in this case. These differences include the lack of a high-affinity thyroid-binding globulin in rats relative to humans (Dohler et al., 1979) that likely affect the turnover of the hormone. With a more rapid turnover of T4 in rats than humans, there is a generalized increased activity of the pituitary–thyroid axis in rats relative to humans, which correlates with increased susceptibility of rats to thyroid gland neoplasia by this MOA. These quantitative differences are discussed in more detail next.

I. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

The postulated mode of action for PB-induced thyroid tumor formation involves the disruption of homeostasis of the thyroid–pituitary axis by an extrathyroidal mechanism. Specifically, PB induces a variety of hepatic xenobiotic-metabolizing enzymes, including UDP-glucuronosyltransferases (UDPGTs). The first step leading to tumor susceptibility is a decrease in circulating T4 (thyroxin) concentrations. Serum levels are decreased because PB induces hepatic UDPGT activity toward T4 and T3 (triiodothyronine), increasing the conjugation of thyroid hormones in liver and enhancing the biliary excretion of the conjugated hormone. In response to the hypothyroid state, TSH synthesis and release is stimulated, and this is the key event that leads to thyroid follicular cell growth and hyperplasia (McClain, 1992; Thomas and Williams, 1999).

In an initiation–promotion model, the thyroid tumor promoting effect of PB was found to be directly proportional to the increased plasma level of TSH. Moreover, dietary supplementation of T4 at dosages that eliminated increased TSH levels completely blocked the thyroid tumor promoting ability of PB (McClain and Rice, 1999). Thus, there is good evidence that the key events already described are directly associated with the thyroid tumor response.

There are very few data available describing the dose-response relationship for alteration of thyroid hormone homeostasis or tumor promotion following PB exposure. In a dietary study, Liu et al. (1995) fed rats PB at levels equivalent to dosages ranging from 46 to 179 mg/kg/day for 15 days. These dosages are substantially higher than the ED_{50} for microsomal enzyme induction (about 11 mg/kg/day; Nims et al., 1993), and a no-effect level was not defined. At all dosages, UDPGT activity toward T4 increased about two- to three-fold. At three days of dosing, serum T4 concentrations were decreased and remained at about a 50% reduction throughout the study. PB treatment also decreased total serum T3 levels by approximately 20% (reported only at 14 days). TSH levels increased about two-fold within one week of dosing, but there was no difference in TSH levels across all dosages of PB tested. More recent evidence suggests that relatively small, dose-dependent increases in TSH levels ($\leq$2-fold) are sufficient to stimulate proliferation of the thyroid follicular cells (Hood et al., 1999). Moreover, it has been suggested that the effects of xenobiotics on the thyroid–pituitary axis demonstrate nonlinear dose-response relationships, as a critical decrease in T4 levels must be reached before TSH will increase in order to compensate (McClain, 1995). In this regard, there is good evidence that there is no risk for tumor induction at dosages that do not alter thyroid–pituitary homeostasis, further supporting the conclusion that increased serum TSH is absolutely necessary to promote thyroid tumor formation.

In response to the hypothyroid state and increased TSH levels, the thyroid gland is stimulated

to produce more T4, and over time, there is compensatory thyroid gland enlargement. This compensatory thyroid gland enlargement has been shown to bring T4 and TSH levels back toward the euthyroid state. Such compensation occurs within three months of exposure to PB (McClain et al., 1989). In contrast, the thyroid gland remains enlarged throughout three months of treatment, suggesting a chronic stimulation of function. Therefore, temporal relationships demonstrate that, although early changes reflect a hypothyroid state, T4 and TSH levels return to near normal levels with more chronic dosing. This compensation, however, is caused by the sustained increased functional activity of the thyroid gland, reflected as a persistent increase in thyroid activity, cell proliferation, and hyperplasia.

One uncertainty that exists in the hypothesis that altered hepatic clearance of thyroxin leads to thyroid tumor development is that not all agents that increase T4 clearance by inducing its glucuronidation also increase TSH levels. For example, with 1 week of treatment, 3-methylcholanthrene (3MC) and polychlorinated biphenyls (PCBs) such as Arochlor 1254 induce the glucuronidation of T4 and markedly increase the biliary excretion of conjugated T4 (Goldstein and Taurog, 1968; Liu et al., 1995). In fact, the biliary excretion of T4-glucuronide is higher with 3MC and PCBs than with PB (Vansell and Klaassen, 2001). However, neither compound caused a compensatory increase in TSH levels (Hood et al., 1999; Lui et al., 1995). Although it has been suggested that these compounds may have additional effects on thyroid hormone catabolism, it is not clear what other events may be influencing the biochemical actions of these compounds. Recent evidence suggests that increased hepatic clearance of T3, the most active form of thyroid hormone, may be necessary to stimulate increased TSH secretion (Vansell and Klaassen, 2002). These authors showed that PCBs and 3MC did not affect T3 glucuronidation and biliary clearance. However, it is presently not known whether agents such as 3MC or PCBs promote thyroid tumor formation in rats. Initation–promotion studies with these compounds would help to eliminate this potential data gap and address the potential differences across the diverse compounds known to alter thyroid homeostasis in rats.

For PB, the available data provide compelling evidence that increased hepatic clearance of T3 and T4 causes chronic stimulation of the thyroid–pituitary axis, seen acutely as an increase in TSH secretion, with long-term stimulation of the thyroid gland as a chronic consequence leading to tumor development. Given that T4 supplementation eliminates the increase in TSH levels and prevents tumor promotion, there is strong evidence for PB that increased clearance of hormones in the liver leads to the long-term stimulation of the thyroid gland and increases the risk of thyroid tumor formation in rats.

II. Are Key Events in the Animal MOA Plausible in Humans?

In order to assess the human relevance of thyroid tumors induced as a result of increased hepatic clearance in rats, it is first necessary to understand the regulation of thyroid hormone homeostasis in humans and to evaluate the role of altered thyroid homeostasis and increased TSH levels as a risk factor for the development of thyroid cancers in humans. Basic physiology indicates that thyroid gland function, including the molecular mechanisms and regulation of thyroid hormone biosynthesis, are similar across species. Moreover, the regulation of pituitary feedback mechanisms that control thyroid gland function are virtually identical across species. Therefore, a decrease in T3 and T4 levels will increase TSH levels in humans.

Sustained alterations of the thyroid–pituitary axis that decrease hormone levels will also stimulate thyroid cell proliferation in humans leading to the formation of goiter. In this regard, there are a number of mechanisms that can affect the thyroid–pituitary axis by mechanims other than increased hepatic clearance of hormones. These disease states or mechanisms include: (1) iodine deficiency; (2) inhibition of the iodide pump; (3) inhibition of thyroid peroxidase required for organification; (4) inhibition of thyroid hormone release; and (5) inhibition of 5′-mono-iodinase (deSandro et al., 1991; Capen, 2001). In all cases, agents that work by any one of the five mechanisms noted may be expected to produce effects in both laboratory animals and humans.

There is also clinical evidence that TSH-induced growth of thyroid epithelial cells is critical to the development of thyroid tumors in humans in certain conditions. For example, in individuals with congenital hypothyroidism caused by mutation in either the thyroid peroxidase or thyroglobulin genes (dyshormonogenesis), TSH levels are elevated from birth, and if not treated, the sustained high levels of TSH lead to multiple benign tumors after several years. Iodide deficiency also stimulates TSH release, leading to goiter which if left untreated can also lead to tumor formation (Thomas and Williams, 1999). These cases represent extreme situations requiring genetic mutations or dietary deficiencies, but

demonstrate that there is some evidence for TSH-stimulated thyroid tumor formation in humans.

III. Taking into Account Kinetic and Dynamic Factors, Is the Animal MOA Plausible in Humans?

There are a number of agents that alter thyroid hormone homeostasis by acting primarily on the thyroid gland (discussed earlier). While it is clear that agents that inhibit organification of iodine (such as propylthiouracil or various sulfonamides), inhibit hormone release (such as lithium), or inhibit 5′-monoiodinase (such as amiodarone or iopanoic acid) can cause hypothyroidism, there is no evidence that these agents increase susceptibility to thyroid cancer (Ron et al., 1987).

PB and other microsomal enzyme inducers do not target the thyroid directly. Rather, the primary effect is on the liver, and increased metabolic enzyme activity indirectly increases the systemic clearance of thyroxin leading to the hypothyroid state and the compensatory increase in TSH**.** The potential for PB or other therapeutic agents that cause or promote thyroid tumors in rats to disrupt thyroid hormone homeostasis in humans has been studied. In human subjects dosed orally with 100 mg PB (approximately 2 mg/kg/day), there was evidence of enzyme induction in the subjects, most notably increased clearance of antipyrine and increased urinary excretion of 6-β-hydroxycortisol. In direct contrast to animals, no changes in thyroid hormones or TSH were noted (Ohnhaus et al., 1981). When PB (100 mg/day) was coadministered with antipyrine (1200 mg/day), serum T4 levels decreased by about 20%. Despite this decrease, however, no change in TSH levels was observed (Ohnhaus and Studer, 1983). Data from other pharmaceutical compounds that induce hepatic microsomal enzymes provide similar evidence, as drugs such as phenytoin, rifampin, and carbamazepine can reduce circulating T4 levels without altering TSH (Curran and DeGroot, 1991). Similarly, proton pump inhibitors (omeprazole, lansoprazole, and pantoprazole), which markedly increase the glucuronidation and biliary excretion of T4, ultimately cause thyroid tumors in rats. The dosages at which the proton pump inhibitors produce these effects are much higher than those used clinically, and clinical data indicate that these drugs produce no changes in thyroid hormones in humans (Masubuchi et al., 1997). Therefore, as a class, agents that produce a hypothyroid state by altering hepatic clearance of thyroxin are readily distinguished from those agents that act directly on the thyroid gland in that the changes in T4 or T3 levels are typically insufficient to increase TSH levels in humans. Collectively, these data also demonstrate the importance of the dose-response relationship in altered homeostasis of the thyroid–pituitary axis. In particular, a critical level of reduction in circulating thyroid hormones is needed to stimulate TSH release. If this critical level is not reached, TSH is not increased, and there is no increased risk of thyroid tumor development.

The decreased sensitivity of the human thyroid–pituitary axis to increased hepatic clearance of thyroxin is not fully understood, but appears to be influenced by several important quantitative differences between rats and humans. These quantitative differences include:

- The half-life of T4 in rats is approximately 12 h, whereas in humans, the half-life is 5–9 days (Dohler et al., 1979). The shorter half-life is likely related to a high-affinity binding globulin for thyroxin that is present in humans but absent in rodents. Specifically, binding of the hormone to thyroid-binding globulin accounts for slower metabolic degradation and clearance.
- Increased turnover and hepatic clearance of T3 and T4 renders the basal activity of the thyroid gland markedly more active in rats than in humans. In the absence of a functional thyroid gland, a rat requires approximately 10 times more T4 than an adult human for full reconstitution (Dohler et al., 1979).
- Constitutive TSH levels are nearly 25 times higher in rats than in humans, reflecting the increased activity of the thyroid–pituitary axis in rats (Dohler et al., 1979; McClain, 1992).

Rats are very susceptible to thyroid neoplasia secondary to hypothyroidism. In particular, modest changes in thyroid hormone homeostasis will promote tumor formation in rats. In contrast, data in humans suggest that prolonged TSH stimulation of the thyroid is unlikely to induce malignant changes (Curran and DeGroot, 1991). This conclusion is also supported by the lack of evidence that patients with Graves disease, where an autoantibody stimulates the TSH receptor, are at an increased risk for developing thyroid cancer (Mazzaferri, 2000).

The key events in rodent thyroid carcinogenesis induced by increased hepatic clearance of thyroxin are summarized in Table 6.

TABLE 6
Comparison of Key Events in Animals and Humans

Key event	Evidence in animals	Evidence in humans
Decreased serum T3 and T4	Observed routinely in studies with PB, dosages studied range from 50 to 175 mg/kg/day	Not observed after PB treatment at clinical dosages of 1–2 mg/kg/day; decreases seen with some other microsomal enzyme inducers
Increased TSH levels	Increased at least two-fold	No changes observed, even when T4 is decreased; no other microsomal enzyme inducers increase TSH levels
Increased TSH promotes thyroid cell proliferation and tumor formation	Proven directly	No association expected given absence of effect on TSH

In support of the biochemical and clinical findings just summarized, there is also epidemiologic evidence that long-term treatment with PB is not associated with increased risk of thyroid tumors. Specifically, there has been an ongoing epidemiologic evaluation of PB-treated epileptic patients to determine whether these patients are at increased risk for any type of cancer. In this population of at least 8000 patients, no increased risk of thyroid tumor development has been observed (Olsen et al., 1993).

IV. Statement of Confidence; Decision Analysis

There is convincing quantitative evidence that rodent thyroid tumors induced by a process involving increased hepatic clearance of hormone and altered homeostasis of the pituitary–thyroid axis are not relevant to humans. Clinical dosages of PB that do cause hepatic enzyme induction are not sufficient to alter T4 homeostasis, and other microsomal enzyme inducers that decrease T4 levels show no evidence of compensatory increases in TSH levels. This conclusion is bolstered by epidemiologic studies with PB that, albeit limited, do not show any increased risk of thyroid cancer, even with chronic treatment. Furthermore, other drugs that induce microsomal enzymes and alter the thyroid–pituitary axis in rodents are also not considered to be human health risks (Curran and DeGroot, 1991). There is also broad-based cellular and biochemical evidence that although thyroid tumors might develop in humans when hormonal imbalances lead to elevated TSH levels, rats are much more sensitive than humans to these perturbations. This sensitivity is likely the result of the rapid turnover of T4 in rats coupled with the higher demand for TSH to maintain thyroid activity.

V. Implications

This case demonstrates the importance of understanding the basic physiology of thyroid hormone regulation and illustrates how qualitatively similar phenomena can be distinguished quantitatively. There is a substantial body of evidence supporting the role of microsomal enzyme induction and increased hepatic clearance of thyroxin leading to altered thyroid hormone homeostasis in rodents. The case provides an example of the level of evidence and completeness of a data set that is needed to address human relevance of carcinogenic responses in animals when tumor susceptibility is determined by quantitative differences in basic physiological processes. It also demonstrates the importance of the dose-response relationship and the concordance of the dose-response relationship with tumor outcome in evaluating a mode of action. Finally, this case is one in which clinical and epidemiological data were available on the chemical of interest, and clinical evaluations were available on functionally similar compounds. These data provided a means to compare and evaluate rodent and human responses and were essential to establishing quantitative differences in tumor susceptibility by this mode of action.

Lois Lehman-McKeeman
Richard Hill

F. MOA: Sustained Cytotoxicity and Cellular Regeneration

Kidney and Liver Tumors Associated With Chloroform Exposure (Case Study 6)

Sustained cytotoxicity and regenerative cell proliferation are key events in the hypothesized modes of action for chemical induction of a range of animal tumors. The example included here is chloroform, which causes liver and kidney tumors in mice and kidney tumors in rats. This case presents an analysis involving several animal tumor types for which chemical-specific data relevant to assessing the weight of evidence of the hypothesized mode of action are considerable. Although the weight of evidence varies, chemical-specific data for key events in the mode of action for formation of these tumors are available from animal studies, and qualitative and quantitative analyses support, with few exceptions, the potential applicability of the animal mode of action in humans. Subject to uncertainties outlined later, the overall weight of evidence indicates that chloroform-induced animal tumors formed as a result of sustained cytotoxicity and cellular regeneration are relevant and useful for evaluating human risk.

I. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

A. Postulated Mode of Action

The hypothesized mode of action for chloroform-related liver and kidney tumors in mice and rats is similar and finds support in histopathologic and metabolic data from several different sources. However, the weight of evidence varies considerably, and, as a result, liver and kidney tumors are addressed separately here.

Considerable information is available concerning the potential mode of induction of these tumors by chloroform. This includes a range of metabolic studies. In addition, while there have been no cancer bioassays in which a range of intermediate endpoints has been investigated, proliferative response in target organs has been examined in numerous investigations following exposure via regimens similar to those in the long-term studies. The histopathology in the target organ for one of the more critical studies has also been reexamined (Hard et al., 2000). These data have been generated to investigate primarily the hypothesized mode of action for tumor induction in rodents for which the requisite precursor steps to cancer are (1) generation of phosgene/HCl by CYP2E, (2) sustained cytotoxicity, and (3) subsequent persistent regenerative cell proliferation.

B. Evidence in Animals/Key Events

Metabolism of chloroform to phosgene, resulting from the oxidative pathway that predominates at low exposures, is believed to be the principal determinant of sustained toxicity and resulting persistent proliferation that is hypothesized to lead to a higher probability of spontaneous cell mutation and subsequent cancer.

Available data indicate that the toxicity of chloroform is attributable to its metabolites. In the liver, for example, both the incidence and severity of toxicity correlate with the level of covalent binding of chloroform metabolites to tissue macromolecules, and phosgene is believed to be quantitatively responsible for the irreversible binding of chloroform metabolites to liver components (Pohl et al., 1980). The extent of chloroform-induced hepatic necrosis also correlates with the extent of covalent binding to protein in male and female rats and in male mice (Ilett et al., 1973; Brown et al., 1974). This covalent binding is more prevalent within the areas of necrosis (Ilett et al., 1973; Tyson et al., 1983), and the association of metabolism with toxicity is further supported by localization of binding to necrotic lesions (Ilett et al., 1973). The results of in vitro studies are consistent, in that irreversible binding to macromolecules in rat and human liver microsomes requires prior metabolism (Cresteil et al., 1979).

Increased covalent binding of chloroform metabolites in the liver also occurs when glutathione is depleted, while some degree of protection is conferred if glutathione or a precursor is administered (Stevens and Anders, 1981). Because covalent binding of a chloroform metabolite with glutathione precedes and becomes maximal prior to the chloroform-induced hepatic cytotoxicity, depletion of glutathione may contribute to the observed cytotoxicity as it does to covalent binding (Stevens and Anders, 1981).

In mice, covalent binding of chloroform to renal proteins and microsomes is correlated with the degree of renal tubular necrosis (Ilett et al., 1973;

Smith and Hook, 1983, 1984). Strain- and sex-related differences in sensitivity of mice to nephrotoxicity are also correlated with the ability of the kidney to metabolize chloroform (Taylor et al., 1974; Clemens et al., 1979; Pohl et al., 1984; Smith et al., 1984; Mohla et al., 1988; Henderson et al., 1989; Hong et al., 1989). In an investigation in F344 rats, however, it was concluded that intrarenal bioactivation of chloroform by cytochrome P-450 did not appear to play a major role in nephrotoxicity (Smith et al., 1985).

The primary, if not only, enzyme catalyzing metabolism at low concentrations of chloroform is cytochrome P-4502E1 (CYP2E1) (Brady et al., 1989; Guengerich et al., 1991).

Regional distribution of lesions in the liver of rats and mice also correlates well with the hepatic distribution of CYP2E1 and glutathione. The highest concentrations of CYP2E1 in both uninduced and induced rat and human liver are present in the centrilobular region (Ingelman-Sundberg et al., 1988; Tsutsumi et al., 1989; Johansson et al., 1990; Dicker et al., 1991). In comparison, concentrations of the phosgene-scavenging agent glutathione in the centrilobular region are only about half those in the periportal region (Smith et al., 1979).

Measures of cytotoxicity include histopathological effects and release of hepatic enzymes and labeling indices as surrogates for regenerative cell proliferation.

1. Key events: Dose-response relationship/temporal association

In all cases where examined, sustained cytotoxicity and cellular proliferation were observed in the liver and kidney of the same strain of mice and rats exposed in a similar manner in short-term studies to concentrations or doses that induced tumors in these organs in cancer bioassays. However, the converse is not always true. Tumors have sometimes not been observed in cases of sustained increases in damage and resulting proliferation in the same strain exposed to similar concentrations in the same manner in shorter term studies, namely, kidney lesions in B6C3F1 mice and F344 rats. These results are consistent with the hypothesis that where chloroform causes tumors, toxicity and reparative hyperplasia are obligatory precursor steps. Tumors would not necessarily be expected whenever there is an increase in cell replication. The multiple susceptibility factors that produce tumors following cytotoxicity will depend on tissue-specific factors and will likely vary between species and strains. For example, in spite of the overt toxicity and sustained increased cell proliferation in the epithelial tissue of the nose in both rats and mice, tumors have not been noted in this tissue in chronic studies, including the inhalation bioassay in which nasal tissues were carefully evaluated (Yamamoto, 1996).

2. Strength, consistency, specificity of association of tumor response with key events

Liver tumors—mice

Liver tumors are observed in B6C3F1 mice following administration of bolus doses by gavage in corn oil (NCI, 1976), but not following administration of the same daily doses in drinking water (Jorgenson et al., 1985). That dose rate is a critical determinant of tissue damage (e.g., being greater following bolus dosing by gavage compared with continuous administration) is consistent with the proposed mode of induction of tumors, with higher bolus doses leading to tissue damage. Doses at which tumors have been observed following administration in corn oil in the cancer bioassay are associated in shorter term studies with sustained proliferative response in the liver of the same strain exposed similarly (Larson et al., 1994b; Pereira, 1994; Melnick et al., 1998). Sustained increases in proliferative response have not been observed following ingestion in drinking water of concentrations that did not induce increases in hepatic tumor incidence in the long-term bioassay (Larson et al., 1994a).

The incidence and severity of hepatic necrosis in the mouse liver have been related to the degree of covalent binding of chloroform metabolites to tissue proteins. The linking of metabolism to toxicity is underscored by localization of covalent binding to the necrotic lesions and the predictable variations in toxic response produced by pretreatment with inducers or inhibitors of cytochrome P-450-mediated metabolism, specifically CYP2E1. There is strong evidence that it is the oxidative metabolites specifically that predominate at low concentration and cause cytotoxicity in the mouse liver. This includes a direct correlation between binding to the polar heads of phospholipid molecules (caused by oxidative metabolites) and protein binding in the liver of the mouse strain in which tumors have been observed (Ade et al., 1994). Particularly strong evidence of the role of CYP2E1 in the induction of mouse liver tumors is also provided by studies in

CYP2E1 null mice. There was no cytotoxicity or cell proliferation in the liver of two strains of CYP2E1 null mice (Sv/129 and B6C3F1 strains) at a concentration that caused severe hepatic lesions in the wild type of either strain (Constan et al., 1999). There is a consistent association between CYP2E1 distribution, chloroform metabolism, pattern of covalent tissue binding, and toxic injury to hepatocytes in mice.

Evidence of concordance between metabolism to reactive intermediates, sustained cytotoxicity, persistent regenerative proliferation, and tumor development in the mouse liver is, therefore, very strong. Indeed, a wealth of information describes a relationship between sustained enhanced proliferative response and induction of liver neoplasia in the strain in which tumors have been observed (B6C3F1 mice).

Renal tumors—mice

Chloroform also induces renal tumors in BDF1 mice following inhalation (Yamamoto, 1996) and in ICI mice exposed by gavage in toothpaste (Roe et al., 1979), although at lower rates than liver tumors. The response is strain and sex specific, occurring only in males.

Evidence of concordance between metabolism to reactive intermediates, cytotoxicity, regenerative proliferation, and tumor development in the mouse kidney, although strong, is not as robust as for the mouse liver, due primarily to the more limited data available on sustained enhanced proliferative response in the strains in which tumors have been observed. Indeed, this is limited to a single study in BDF1 mice, in which there was an increase in labeling index in the kidneys of males but not females at concentrations that induced renal tumors in this strain in the long-term inhalation bioassay (Templin et al., 1996b; Yamamoto, 1996). The available data concerning the relationship between sustained cellular proliferation and induction of renal tumors in another strain (B6C3F1) of mice indicate that sustained proliferative response is not always associated with tumors. In this strain, in shorter term studies, there were sustained proliferative responses at doses at which kidney tumors were not observed in the relevant cancer bioassays following exposure by gavage both in corn oil and drinking water (National Cancer Institute [NCI], 1976; Jorgenson et al., 1985; Larson et al., 1994a, 1994b).

In mice, covalent binding of chloroform to renal proteins and microsomes is correlated with the degree of renal tubular necrosis, with strain and sex differences in sensitivity to nephrotoxicity being correlated with the ability of the kidney to metabolize chloroform. Similar to the liver, there is strong recent evidence that it is the oxidative metabolites specifically that predominate at low concentration and cause cytotoxicity in the mouse kidney. This includes a direct correlation between binding to the polar heads of phospholipid molecules (caused by oxidative metabolites) and protein binding in the kidney of DBA/2J mice (Ade et al., 1994). Particularly strong evidence of the CYP2E1's role in the induction of mouse renal tumors is also provided by recent studies in CYP2E1 null mice. Neither cytotoxicity nor cell proliferation was observed in the kidney of two strains of CYP2E1 null mice (Sv/129 and B6C3F1 strains) at a concentration that caused severe hepatic lesions in the wild type of either strain (Constan et al., 1999).

Renal tumors—rat

The weight of evidence for the hypothesized mode of induction of tumors in the rat kidney is considerably less than that for the mouse liver and kidney, due primarily to limited data on intermediate endpoints in the only strain (Osborne-Mendel) in which increases in kidney tumors have been observed. These increases have been reported following exposure via gavage both in corn oil and drinking water (NCI, 1976; Jorgenson et al., 1985). There are also few identified data on the relationship between the metabolism of chloroform and induction of renal lesions in rats. In the F344 rat, there were sustained increases in proliferative response in shorter term studies following administration of doses similar to those that induced tumors in Osborne-Mendel rats following administration by gavage in corn oil but not following ingestion in drinking water (Larson et al., 1995a, 1995b). However, there are no bioassays in this strain following ingestion for direct comparison with these results. Sustained increases in labelling index were observed in the proximal tubules of F344 rats exposed to daily doses of 30 ppm (147 mg/m^3) and greater and at 90 ppm (441 mg/m^3) and greater at 5 days per week (Templin et al., 1996a). However, increases in kidney tumor incidence were not observed in this strain exposed to up to 90 ppm (441 mg/m^3) for 5 days per week in the only inhalation cancer bioassay (Yamamoto, 1996).

Based on studies conducted primarily in F344 rats in which tumors have not been observed, a mode of action for carcinogenicity in the kidney observed

in the carcinogenesis bioassay in Osborne-Mendel rats based on sustained cytotoxicity and persistent tubular cell regeneration is, therefore, plausible. For Osborne-Mendel rats, the results of reanalyses of the original renal tissues (Hard et al., 2000) from both the drinking water bioassay (Jorgenson et al., 1985) and the gavage study (NCI, 1976) have been critical. They provide strong support for the contention that the mode of induction of these tumors is consistent with the hypothesis that sustained proximal tubular cell damage is a requisite precursor lesion for chloroform-induced tumors.

3. Biological plausibility and coherence of the database

The organs in which chloroform-induced cytotoxicity and proliferative lesions are observed (liver, kidney, and nasal passages) correlate well with the distribution of CYP2E1 both across and within species (Löfberg and Tjälve, 1986). This consistent pattern of response to chloroform across species and organs supports a conclusion that chloroform-induced neoplasia is dependent on sustained cytotoxicity coupled with persistent regenerative cell proliferation. This is further supported by the considerable weight of evidence indicating that chloroform is not genotoxic, with unconvincing evidence for direct DNA reactivity. Due principally to limitations of the available data, though, weak genotoxicity in the rat cannot be precluded, which detracts somewhat from the weight of evidence in this species, although it is unknown whether this might be a result of secondary effects on DNA.

The hypothesized mode of carcinogenesis for chloroform is in keeping with the growing body of evidence supporting the biological plausibility that prolonged regenerative cell proliferation can be a causal mechanism in chemical carcinogenesis. This has been addressed in numerous articles, including Ames and Gold (1990, 1996), Cohen and Ellwein (1990, 1991, 1996), Preston-Martin et al. (1990), Ames et al. (1993), Tomatis (1993), Cohen (1995a), Cunningham and Matthews (1995), Butterworth (1996), Farber (1996), and Stemmermann et al. (1996). Enhanced cell proliferation can lead to an increased frequency of spontaneous genetic damage either through errors that result from the infidelity of DNA replication or through the increased conversion of endogenous DNA changes into heritable genetic changes (Cohen and Ellwein, 1990, 1991, 1996; Ames et al., 1993; Cohen, 1995a). Additionally, during periods of cell replication, heritable nonmutagenic modifications of the genome may occur that may lead to changes in gene expression, contributing to carcinogenesis (U.S. EPA, 1996). This view that cell proliferation is a risk factor for carcinogenesis is not universally accepted, because strict correspondence between increased cell turnover and carcinogenic response is not always demonstrable (Melnick, 1992; Farber, 1996). However, as indicated earlier, in view of the complex interplay of factors involved in the carcinogenesis process, it is not surprising that acute measures of cell proliferation do not always indicate a one-to-one correlation. Among the factors to be considered are the kinetics of DNA adduct formation and repair; the balance between cell proliferation, differentiation, and death; proliferation in the target cell compartments compared with that of nontarget cells; and the consequences of overt tissue toxicity.

4. Alternate modes of action

While the evidence is fairly convincing that chloroform acts principally through cytotoxic effects of phosgene and other products of oxidation, other possibilities involve mutagenicity. One possibility is that the effects of chloroform are a composite of metabolites from both oxidative and reductive pathways contributing to toxicity and carcinogenicity. However, several observations strongly support the predominant role of oxidative pathways in chloroform toxicity and make any significant role of reductive metabolism highly unlikely. First, the macromolecular binding following administration of chloroform represents only a very small portion of the delivered dose. Second, the mechanisms of action related to the nature of the necrotic lesion, the time course of injury after single doses, and the differences in cumulative damage on multiple exposures are very different for chloroform and carbon tetrachloride, the latter a compound for which the free radical (reductive) pathway is causative for toxicity. In addition, carbon tetrachloride, which is largely metabolized to a free radical, is not itself mutagenic. Based on these considerations, it was concluded that free radicals do not play a significant role in the toxicity or carcinogenicity of chloroform.

Another possibility is that minor pathways, associated with glutathione conjugation, produce mutagenic metabolites, as is believed to be the case for dichloromethane. However, there is little evidence for a significant direct conjugation pathway for chloroform. In studies with *Salmonella* tester strains with glutathione transferase T1-1 inserted

into the bacterial genome and expressed during testing, a small increase in mutagenic activity (less than a factor of 2) was noted for chloroform at very high doses, even though positive controls with methylene chloride and bromochloromethane produced much larger responses (Pegram et al., 1997). Neither of these potential modes of action is believed to play a significant role in the observed toxicity and carcinogenicity of chloroform, although further investigation of weak genotoxicity in the rat is desirable.

C. Conclusion: Assessment of Postulated Modes of Action in Animals and Statement of Confidence

In summary, then, chloroform has induced liver tumors in mice and renal tumors in mice and rats. The weight of evidence—genotoxicity, sex and strain specificity, and concordance of sustained cytotoxicity, persistent regenerative proliferation, and tumors—is consistent with the hypothesis that marked cytotoxicity concomitant with a period of sustained cell proliferation likely represents a secondary mechanism for tumor induction following exposure to chloroform. This is consistent with a nonlinear dose-response relationship for induction of tumors. This cytotoxicity is primarily related to oxidation rates of chloroform to reactive intermediates, principally phosgene and hydrochloric acid. The weight of evidence for this mode of action is strongest for hepatic and renal tumors in mice and more limited for renal tumors in rats.

The evidence that supports an obligatory role of sustained cytotoxicity in the carcinogenicity of chloroform is considerable. Indeed, there are few compounds for which the supporting database in this regard is as complete, consistent and cohesive as it is for chloroform. Although there are some uncertainties, the weight of evidence is strongest for hepatic and renal tumors in mice. The evidence is more limited for renal tumors in rats, primarily due to the relative paucity of data in strains where tumors have been observed. Other data gaps relate to metabolism and intermediate endpoints and the relationship between them. Uncertainty could be reduced by additional information on metabolism, cytotoxicity, and proliferative response in the strain in which tumors were observed (i.e., Osborne-Mendel rats) following long-term exposure to chloroform. Additional data on metabolism and chronic (e.g., 2-year) cytotoxic/proliferative response in the kidneys of F344 rats could also contribute to greater confidence in the hypothesized mode of action.

II. Are Key Events in the Animal MOA Plausible in Humans?

A. Comparative Analysis of Key Events

The comparison of Tables 7 and 8 succinctly illustrates the nature and relative weight of evidence for the hypothesized mode of action of chloroform in humans as well as experimental species

In general, chloroform elicits the same symptoms of acute toxicity in humans as in experimental animals; target organs are also similar. For example, there have been infrequent reports of renal tubular necrosis and renal dysfunction resulting from the use of chloroform as an anesthetic (Kluwe, 1981). Liver toxicity due to occupational exposure to chloroform has also been reported at concentrations in the range of 80–160 mg/m^3 (with an exposure period of less than 4 months) in one study and in the range of 10–1000 mg/m^3 (with exposure periods of 1–4 years) in another study. The mean lethal oral dose for an adult is estimated to be about 45 g, but there are large interindividual differences in susceptibility (World Health Organization [WHO], 1994).

Available epidemiological data do not allow conclusions with respect to the potential carcinogenicity of chloroform in humans. Some reports indicating an association between exposure to disinfection by-products (DPBs) in drinking water and increased risks of bladder cancer fulfill, in part, traditional criteria of causality. However, some inconsistencies in reported differences between men and women and between smokers and nonsmokers are difficult to explain. Moreover, it is not possible to attribute these excesses specifically to chloroform (ILSI, 1997); indeed, due to the relative paucity of exposure information in relevant studies, the sources of increased relative risks are unclear. Specific risks may be due to other DBPs, mixtures of by-products, other water contaminants, or other factors for which chlorinated drinking water or trihalomethanes (THMs) may serve as a surrogate (WHO, 2000).

The information summarized in the comparison tables leads to the conclusion that the weight of evidence for the hypothesized mode of induction of tumors (i.e., metabolism by the target cell population, induction of sustained cytotoxicity by metabolites, and subsequent persistent regenerative cell

TABLE 7
Key Events in Animals and Humans—Liver Tumors

Key event	Animals	Humans
Generation of phosgene/HCL by CYP2E1	Incidence/severity of toxicity correlate with covalent binding of metabolites in rats and mice, more prevalent in necrotic lesions	Irreversible binding to macromolecules in human liver microsomes requires prior metabolism; PBPK model based on human physiological parameters and metabolic parameters in vitro in eight human liver samples
Cytotoxicity	In all cases where examined, sustained cytotoxicity (as measured by histopathological effects and release of hepatic enzymes) in the liver of mice at doses that induce tumors	Liver also a target organ in humans based on reports of effects associated with occupational exposure
Regeneration/proliferation	In all cases where examined, persistent regenerative proliferation (as measured by labeling indices) in the liver of mice at doses that induce tumors	No data
Tumors	Mice	Inadequate epidemiological data

proliferation) is greatest for liver and kidney tumors in mice, followed by kidney tumors in rats. Though data in humans are limited, based on expected similar response in humans and in the absence of data to the contrary, the mode of action for chloroform-related animal tumors is considered to be qualitatively applicable to humans. Available data confirm that target organs in populations exposed occupationally to high concentrations are similar to those in experimental animals (i.e., the kidney and liver).

III. Taking into Account Kinetic and Dynamic Factors, Is the Animal MOA Plausible in Humans?

Other data on the human relevance of the hypothesized mode of action address quantitative variations in rates of metabolism to the putatively toxic metabolite in target organs. Quantitative variations in response between animals and humans for the hypothesized mode of induction of tumors are likely to be a function primarily of variations in rates of metabolism to phosgene in the target tissue. The rates of formation of reactive metabolites of chloroform (namely phosgene) in animals and humans have been estimated pharmacokinetically based on models that include specific parameters related to metabolic rates, enzyme affinities and enzyme tissue distribution. The most refined animal model features two-compartment absorption (stomach and intestinal tract) and subdivision of the liver and kidney compartments into regions of high and low metabolic activity (ILSI, 1997). This "hybrid" animal model has been revised and extended to humans (ICF Kaiser, 1999).

For the human model, the physiological and anatomical parameters were derived from Brown et al. (1997) with the exception of the ventilation rate and cardiac output, which were related to an assumed breathing rate of 23 m^3/day (Health Canada, 1994). Liver tissue subvolumes were assumed to be the same as in the rat, based on Tsutsumi et al. (1989) and Buhler et al. (1992), while kidney was subdivided into a 70:30 cortex:noncortex ratio as described by the International Commission on Radiological Protection (ICRP, 1992). Human metabolic parameters were taken from Corley et al. (1990); these had been determined in vitro in eight human liver samples. Kidney rate constants were based on the relationship of activity observed in the microsomal fraction of kidneys to the activity observed in the microsomal fraction of the liver based on in vitro results reported by Corley et al. (1990) but supported by data on metabolism of two known substrates of CYP2E1 by microsomal fractions of the kidney and liver from 18 humans (Amet et al., 1997).

TABLE 8
Key Events in Animals and Humans—Kidney Tumors

Key event	Mice	Rats	Humans
Generation of phosgene/ HCL by CYP2E1	In mice, strain- and sex-related differences correlate with metabolism; necrosis corrleates with the degree of covalent binding	Few such data for rats and in F344 rats, nephrotoxicity not correlated with bioactivition	Quantitation in PBPK model based on human physiological parameters and activity in the microsomal fraction of kidneys to that in the microsomal fraction of the liver in vitro supported by data on metabolism of two known substrates of CYP2E1 by microsomal fractions of the kidney and liver from 18 humans
Cytotoxicity	In mice, in all cases where examined, sustained cytotoxicity (as measured by histopathological effects and release of hepatic enzymes), at doses that induced tumors	In rats in critical bioassay, cytotoxicity based on histopathological reexamination	Kidney also a target organ in humans based on reports of renal effects resulting from anaesthetic use of chloroform
Regeneraton/ proliferation	In all cases where examined, persistent regenerative proliferation (as measured by labeling indices) in the kidney of mice at doses that induce tumors, though data are less than for liver	Studies in rats restricted to those in a strain where tumors have not been observed	No data
Tumors	Mice	Rats	Inadequate epidemiological data

Results from the human model were compared with data on total metabolized parent and exhaled chloroform reported by Fry et al. (1972) in an investigation in which chloroform was administered to male and female volunteers in olive oil or gelatin capsules. Exhaled chloroform was measured for up to eight hours following exposure, and the total percentage of the dose exhaled unchanged was calculated by extrapolation to infinite time. Human model simulations conducted using a single-compartment description of oral uptake were closer to the observations of Fry et al. (1972) than those estimated using a multicompartment description. Therefore, while a multicompartment description was necessary in the rat model, a single-compartment description of oral uptake was used in estimating human equivalent concentrations.

Quantitative variations in delivered dose to the target organ predicted by the physiologically based pharmacokinetic model for dose-response analysis (i.e., kidney) for mice, rats, and humans are consistent with the magnitude of difference expected based on species variations in metabolic rates, with the value for rats being approximateily 15-fold greater than that for humans at the same dose[3] (0.491 vs. 0.0335 ϕg/h/L for the mean or maximum rate of metabolism per gram kidney cortex volume, assuming continuous ingestion of 1 ϕg/kg/day in drinking water).

[3]Yates et al. (1994) also reported single- and double-strand breaks in plasmid DNA incubated with 2-cyanoethylene oxide.

IV. Statement of Confidence; Decision Analysis; Implications

There is a high degree of confidence in the weight of evidence for an obligatory role for cytotoxicity in the carcinogenicity of chloroform, including a nonlinear dose-response relationship for tumor induction in animals. Because histopathologic effects, rather than biochemical effects such as increases in urinary enzymes, are the most sensitive indicator of damage, it is difficult to envisage other information on markers of effect that might reasonably be collected in additional study in humans. For example, scientists are unlikely to recommend biopsies for this purpose.

Although toxicokinetic data only are available for quantifying relative sensitivity to chloroform in humans, expected similarities in the mode of action in animals and humans give little reason to expect qualitative differences in the response of human tissues to this chemical. Quantitative variations in chloroform metabolism in animal and human target cell populations have been characterized in a physiological toxicokinetic model. Variations in metablic parameters, particularly in the kidney for humans, had the greatest impact in the sensitivity analysis. Data from in vitro studies exposing target tissue from both rats and humans to the putatively toxic metabolite of chloroform could provide additional information on relative sensitivity.

If performed on tissues from a number of individuals, additional in vitro data on the metabolism of chloroform in the human kidney and liver would be useful not only to reduce uncertainty in these values but, potentially, to address variability across the human population. In particular, it would be desirable to clarify whether the same metabolic pathways contribute to the potential for cytotoxicity in rodents and humans, specifically with respect to CYP2 E1 and other P-450 isozymes.

The case presents considerable evidence for an obligatory role for cytotoxicity in the carcinogenicity of chloroform, consistent with a nonlinear dose-response relationship for tumor induction in animals and, by inference, in humans. Unfortunately, few compounds will have a supporting database that is as complete, consistent, and cohesive for mode of action and human relevance analysis as chloroform. While qualitatively applicable to humans, quantitative variations in response between animals and humans for the hypothesized mode of action are likely due to variations in rates of metabolism to phosgene in the target tissue. These quantitative variations between species in metabolism of chloroform by the target cell population have been characterized in a physiological toxicokinetic model. For this model, among those parameters considered in the sensitivity analysis to have most impact on output, uncertainty was greatest for the metabolic parameters, particularly in the kidney for humans.

For chloroform, the data for analyzying human relevance are principally compound specific, including kidney and liver toxicity following exposure at high levels during use of chloroform as an anesthetic or following occupational exposure. Quantitative scaling to humans in the PBPK model was based on more generic information on physiological parameters in humans and human metabolic parameters in vitro, supported by metabolism of two known substrates of CYP2E1 by microsomal fractions of the kidney and liver from 18 humans (Amet et al., 1997).

Although this case study focuses on mode of action analysis, it also provides information useful for dose-response analysis. Specifically, if data on precursor events are inadequate, the incidence of obligatory noncancer precursor events (cytotoxicity and sustained regenerative proliferation) from interim kills in the critical cancer bioassay or tumors would be most useful for the dose-response analysis in a risk assessment for chloroform. In addition, in view of the weight of evidence for the role of the oxidative metabolites of chloroform in the induction of requisite damage and resulting tumors, dose response might optimally be expressed as amounts or rates of formation of reactive metabolites produced per volume of tissue in the critical organ.

M.E. Meek

G. MOA: Urinary-Tract Calculi

Urinary Bladder Tumors Associated with Exposure to Melamine (Case Study 7)

Urinary-tract calculi are produced by a variety of substances in rats or mice and are associated with a significant incidence of bladder tumors. Some examples are uracil, 4-ethylsulfonylnaphthalene-1-sulfonamide, fosetyl-A1, and melamine. Similarly, urinary-tract calculi occur relatively commonly in

humans, occasionally related to the ingestion of specific chemicals, including certain sulfonamides and acetazolamide. However, epidemiologic evidence shows only a weak association between calculi in humans and the development of urinary bladder tumors. This finding raises questions about the relevance for human risk assessment of chemically induced bladder calculi and related tumors in laboratory animals.

This case study on melamine illustrates the importance of mode of action information in making decisions on the use of these animal tumors for human risk assessment, with special emphasis on the role of information from studies on other chemicals and the impact of information on the potential for human exposure (U.S. EPA, 1988; IARC, 1999a).

Melamine, a synthetic chemical used in the manufacture of resins, is not metabolized and is considered non-DNA-reactive (NTP, 1983; IARC, 1999a). Dietary administration of melamine produces bladder tumors in male rats but not in female rats or mice of either gender (NTP, 1983). No epidemiologic or toxicity data are available on the consequences of melamine exposure in humans (IARC, 1999a). In the absence of such data, information on the mechanism of action in animals and in humans becomes an important asset in evaluating the applicability of the animal tumor data to human risk assessment. Specifically, melamine is postulated to produce bladder tumors in rats secondary to the formation of urinary tract calculi producing toxicity and regeneration of the bladder epithelium (IARC, 1999a). The calculi are composed of melamine and uric acid. The sequence of events in bladder tumorigenesis involves administration of high doses sufficient to precipitate melamine in the urine leading to the formation of melamine/uric acid-containing calculi, which cause urothelial toxicity and consequent regeneration, and ultimately the formation of tumors (IARC, 1999a). Although such a sequence is plausible in humans, it is recognized to be a high-dose-only phenomenon so exposure considerations are critical in evaluating potential risk to humans (IARC, 1999a).

The case represents a relevance analysis based on possible qualitative similarities between animal and human responses, but also likely quantitative differences in sensitivity to the potential carcinogenic effects of calculi once they are present and marked quantitative differences between exposures to laboratory animals and expected human exposure.

I. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

A. A Well-Defined Mode of Action

In the National Toxicology Program (NTP) bioassay on melamine administered in the diet, bladder tumors were produced in male F344/N rats but not in female rats or in male or female B6C3F1 mice, although single transitional-cell papillomas were observed in the low (4500 ppm) and high (9000 ppm) dose groups in female rats (NTP, 1983). In the male rats, except for one rat, all rats with bladder carcinomas also had a bladder calculus. In the chronic bioassay, none of the female rats had bladder calculi. No bladder tumors were seen in male or female mice although there were cases of epithelial hyperplasia. High incidences of bladder calculi were observed in male mice but not in female mice. In subchronic studies (13 weeks), bladder calculi were readily formed in male rats, but less commonly in female rats. Doses required to form calculi in female rats were considerably higher than in males. Similarly, the doses required to form calculi in mice were significantly higher than in the rats, and there was a lower incidence of calculi in the subchronic study than in the chronic bioassay. In the subchronic study, urothelial ulceration and inflammation were observed, usually seen in animals with calculi. However, several animals with calculi did not have ulcerations or cystitis. No information on melamine urinary concentrations in the different species and genders was available (IARC, 1999a).

In an experiment involving exposure to male rats for 36 weeks followed by 4 additional weeks of observation, 1% and 3% melamine in the diet produced urinary calculi with a clear dose response, and bladder tumors, including papillomatosis, papillomas, and carcinomas, were induced (Ogasawara et al., 1995). When rats were cotreated with 5 or 10% NaCl, there was an increased ingestion of water and a consequent increased urinary volume, presumably associated with a dilution of urinary melamine. This was associated with a decrease in bladder calculi and bladder tumors in the rats administered 1% melamine, but not significantly with 3% melamine. Based on these studies with melamine, it has been concluded that bladder tumors are associated with administration of high doses of melamine, and that the tumors are related to precipitation of urinary melamine with the formation of urinary tract calculi (IARC, 1999a). Although the correlation between

calculi, ulceration, hyperplasia, and bladder tumor formation has not been 100%, explanations based on studies with other chemicals, such as uracil, that produced urinary-tract calculi have been advanced to explain the discrepancies (Clayson et al., 1995; Fukushima et al., 1992; Otori et al., 1997; Shirai et al., 1989).

It appears that ulcerations secondary to calculus formation occur relatively rapidly and are repaired, even with continued presence of the calculus (Clayson et al., 1995; IARC, 1999b). It is thus not unusual to see extensive proliferation of the bladder epithelium in the presence of calculi at later time points, such as those seen in the experiments with melamine, without an associated ulceration or intense inflammatory response. Chronic inflammation is frequently present, however. Similarly, correlation between the presence of calculi and tumors at later time points is not 100%. This has been explained by the loss of calculi during the experiment, either by dissolution or, more likely, spontaneous evacuation from the urinary tract. For some chemicals, such as fosetyl-Al, rats observed for relatively short periods of time have nearly an 100% incidence of urinary tract calculi, whereas at later time points when tumors were observed, there was a much lower incidence of calculi (Clayson et al., 1995; IARC, 1999b; Rodent Bladder Carcinogenesis Working Group, 1995).

With urinary tract calculi in general, urothelial toxic and proliferative responses have been greater in male rats than in female rats, and generally greater in rats than in mice (Clayson et al., 1995; IARC, 1999b; Rodent Bladder Carcinogenesis Working Group, 1999). This was seen with melamine (IARC, 1999a). Also, the proliferation in response to the presence of calculi varies between species and sexes, generally for unknown reasons. With melamine, rats more commonly developed bladder tumors in response to the presence of calculi whereas the mice only developed hyperplasia and at lower incidences than for bladder lesions in rats (IARC, 1999a). As the studies with melamine demonstrate, the mere presence of a calculus in the urinary tract does not assure the ultimate formation of a bladder tumor (Clayson et al., 1995; IARC, 1999b; Rodent Bladder Carcinogenesis Working Group, 1995). Numerous factors appear to affect the proliferative response to the calculi, including size, number and coarseness of the calculus surface (Clayson et al., 1995; IARC, 1999; Rodent Bladder Carcinogenesis Working Group, 1995). Even with pellets surgically implanted into the bladders of mice, incidences over a 2-year time span are significantly less than 100%, as demonstrated by Jull (1979) utilizing paraffin wax pellets. Approximately half of the mice developed carcinomas after 2 years, while nearly all rats with surgically implanted pellets have tumors by 12–18 months (Bryan, 1969).

With respect to melamine, the association between the formation of the urinary-tract calculi and the ultimate development of bladder tumors in rats is strongly correlated and is a consistent observation in different studies (IARC, 1999a). In experiments performed to date, the observation is consistent, and the response to melamine is specific to formation of calculi and development of urinary bladder tumors. No other tumors are induced. When melamine was administered on the skin, initiating activity was not detected. No evidence of other toxicity or carcinogenic effects has been identified.

B. Statement of Confidence: Supporting and Limiting Factors

The biological plausibility of any postulated mode of action in animals depends in part on additional considerations such as dose-response and temporal relationships, uncertainties, and the likelihood of alternative modes of action.

For melamine, the data just summarized strongly support a correlation between doses leading to calculi, urinary bladder hyperplasia, and bladder tumor formation. Urinary calculi occur relatively uncommonly in rodents except for a few specific strains. The studies with melamine strongly support a temporal relationship between the formation of urinary-tract calculi leading to urothelial toxicity and ulceration, regeneration, and ultimately bladder tumor formation. Studies with other substances, particularly uracil, have provided data in support of this sequence of events in greater detail (Clayson, 1979; Cohen, 1995b; Fukushima et al., 1992; IARC, 1999b; Kagawa et al., 1992; Otori et al., 1997; Shirai et al., 1989). Also, melamine is considered non-DNA-reactive (IARC, 1999a). No modes of action other than calculi have been postulated (IARC, 1999a).

At the same time, although the correlation for melamine between calculi and bladder tumors has been strong, and the dose-response between sexes and species correlates with the response for both calculi and tumors, the correlation has not been 100%. As indicated earlier, this is likely due to numerous factors including the ability of rodents to eliminate bladder stones so that they are not present at the time of observation of the bladder

tumor (Clayson et al., 1995; IARC, 1999b, 1999a). To show a stronger correlation between the early events of calculus formation, ulceration, and proliferation, evaluations of earlier time points are necessary. However, given the more detailed observations with other chemicals and the similarities in observations with melamine relevant to these chemicals, the association between calculi and bladder tumor formation with melamine appears strong. More information about urinary melamine concentrations with different doses and treatment protocols would also be useful in correlating high exposure levels to calculus formation.

In summary, based on the close correlation between calculi and the development of bladder tumors in rodents administered melamine and, separately, a large number of other chemicals, the animal mode of action for bladder tumors related to urinary-tract calculi has become generally accepted. This has also been demonstrated utilizing surgically implanted pellets composed of various materials, such as paraffin wax or cholesterol. In view of the information available for both melamine and other chemicals producing urinary-tract calculi and bladder tumors in laboratory animals, the overall biological plausibility and coherence of the postulated mode of action in animals is strong. Based on this same information, bladder tumors produced by these agents in animals are considered a high-dose-only phenomenon.

II. Are Key Events in the Animal MOA Plausible in Humans?

For melamine itself, there is essentially no information with respect to human toxicity (IARC, 1999a). It does not appear that melamine is metabolized, and most ingested melamine is rapidly excreted in the urine unchanged (Mast et al., 1983; Worzalla et al., 1974). Furthermore, there are no structure–activity relationships for melamine regarding carcinogenesis. Assuming comparable animal and human modes of action, for melamine to produce bladder cancer in humans, adequate exposures must occur to generate a sufficiently high concentration of melamine in the urine for precipitation to occur (Heck and Tyl, 1985; Melnick et al., 1984; U.S. EPA, 1984, 1988; IARC, 1999a). Such high exposures to melamine are not reasonably expected to occur in humans now or in the future (IARC, 1999a). If the calculi do not form, the chemical is not expected to be associated with a carcinogenic risk by this mode of action (Clayson et al., 1995; IARC, 1999a, 1999b; Rodent Bladder Carcinogenesis Working Group, 1995).

In the absence of human data specific to melamine, other information on human carcinogenesis, the biology of the target organ and its response to insult, relevant risk factors, and other considerations can contribute to understanding the mode of action in humans. Although calculus formation in humans has not been demonstrated and is not anticipated for melamine, humans form urinary calculi in response to exposure to other chemicals (Burin et al., 1995; Clayson et al., 1995; IARC, 1999b). Calculi can form either from the administered substance or its metabolites, or from endogenous substances such as calcium oxalate or calcium phosphate, which are excreted at high concentrations because of physiologic alterations resulting from administration of the test substance (Clayson et al., 1995). For melamine in rodents, the calculi are composed of the parent substance, melamine, and an endogenous substance, uric acid (IARC, 1999a).

The relationship of urinary-tract calculi in general to the development of bladder cancer in humans remains undetermined, although the evidence suggests that there is a small relative increased risk associated with the long-standing presence of calculi in the development of bladder cancer (Burin et al., 1995; IARC, 1999b; Rodent Bladder Carcinogenesis Working Group, 1995) (Table 9). The urothelium in humans shows similar differentiation as in rodents, with basal cells, intermediate cells, and large umbrella-like superficial cells (Oyasu, 1995). The specialized asymmetric unit membrane of the luminal surface is the same and is composed of a strongly conserved set of proteins referred to as uroplakins. Similar to rodent models, the postulated sequence of events is the development of urinary tract calculi, ulceration, regeneration, and the ultimate development of bladder tumors (Clayson et al., 1995; IARC, 1999b). As in rodents (Shirai et al., 1989), if the calculus is removed, the epithelial changes appear to be reversible in humans, at least based on cytologic analyses (Beyer-Boon et al., 1978; Highman and Wilson, 1982). In rodents, the tumors that develop are usually urothelial (transitional) cell carcinomas, which have a pathogenesis similar to that seen in humans (Clayson et al., 1995; IARC, 1999b; Oyasu, 1995). However, a large proportion of tumors in humans associated with calculi, like other causes of inflammation, are squamous-cell carcinomas. Nevertheless, several calculi-associated tumors are urothelial (transitional) cell carcinomas.

There are several confounding factors in evaluating the relationship between calculi and bladder

TABLE 9
Comparative Analysis of Key Events in Animals and Humans for Calculi

Key event	Evidence in animal	Evidence in human
1. Urinary concentration adequate for precipitation	Yes, at high dose exposures	Potential
2. Formation of calculi	Yes, at high dose exposures	Potential
3. Urothelial damage	Yes	Yes
4. Urothelial regeneration	Yes	Yes
5. Urothelial tumor formation	Yes, occurs at high incidence	Yes, relative risk increased by up to twofold

tumors in humans. Urinary calculi actually form relatively commonly in humans, estimated at 2–3% of the population in the United States and Europe (Hiatt et al., 1982). However, based on the anatomy of the urinary tract in humans and the fact that they are upright, bipedal organisms, calculi generally are not present in the human urinary tract for long periods of time (Burin et al., 1995; Rodent Bladder Carcinogenesis Working Group, 1995). They are either quickly, spontaneously voided or they cause obstruction. Obstruction is associated with pain and either surgical or lithotriptic removal of the calculi. In contrast, rodents are horizontal quadripeds, so calculi can remain in the dome of the bladder without producing complete obstruction (Clayson, 1979; Clayson et al., 1995).

There are a few circumstances, namely, bladder diverticuli, neurogenic bladder, and staghorn renal pelvic calculi, where calculi can be present in the human urinary tract for long periods of time (Burin et al., 1995). In such circumstances, some studies, but not all, have demonstrated a slight, increased relative risk for development of bladder cancer. However, in most of these cases, the calculi are associated with bacterial infection, a known risk factor for development of bladder cancer. Thus, whether the calculus itself or the bacterial cystitis, or the two together are the offending agent cannot be determined.

Several of the substances known to produce calculi at high concentrations in rodents and/or humans are naturally occurring, some of which are essential ingredients for life, such as calcium, uric acid, uracil, and cysteine (Clayson et al., 1995; IARC, 1999b; Rodent Bladder Carcinogenesis Working Group, 1995). However, these only pose a potential carcinogenic risk to humans (albeit small) when urinary concentrations are adequate to produce calculi.

Thus, the key events in the mode of action of calculi and bladder cancer are qualitatively applicable to humans (Table 9).

III. Taking into Account Kinetic and Dynamic Factors, Is the Animal MOA Plausible in Humans?

The absence of epidemiologic data from human populations exposed to melamine or information on human toxicity prevents direct comparison between animals and humans. There are no structure–activity relationships known for melamine with respect to bladder carcinogenesis. The triazine ring is generally not metabolized, and triazine compounds are not known to be carcinogenic (Mast et al., 1983; IARC, 1999a; Worzalla et al., 1974). Reactivity with macromolecules is unknown, but there is no evidence that melamine reacts with nucleic acids. Interactions with proteins are also unknown. Protein interactions, if they existed, could potentially influence formation of urinary tract calculi (Clayson et al., 1995; Cohen, 1995b). Kinetic data are relevant as they pertain to the excretion of adequate concentrations of the critical components for the formation of calculi. Such data are not available in animals or humans.

It remains unclear whether urinary tract calculi alone pose a carcinogenic risk to humans or whether it requires the presence of bacterial cystitis as a contributing or independent risk factor. For calculi in general, there are several differences between rodents and humans that affect potential risk (Burin et al., 1995; Rodent Bladder Carcinogenesis Working Group, 1995). In all species tested, a sufficient urinary concentration must be attained for precipitation to occur. This can also be influenced by other urinary factors such as overall osmolality, protein

concentration, and inhibitors such as citrate chelating calcium (Clayson et al., 1995; Cohen, 1995b). However, actual data evaluating factors influencing precipitation of specific chemicals are generally unavailable, as is the case for melamine. Also, male rats and mice more readily form calculi than females, presumably because of the much greater urinary concentration of protein in males, but possibly related to anatomic differences in the urethra.

Based on stature and anatomy, rodents are more likely to retain calculi for longer periods of time than humans, providing a longer exposure to the toxic stimulus (Rodent Bladder Carcinogenesis Working Group, 1995). Thus, humans are less at risk than rodents of developing bladder tumors if calculi actually form. If adequate levels of exposure to a chemical occur to produce urinary calculi, based on the totality of the evidence there is likely a small increased risk of developing bladder cancer in humans. Thus, for a given chemical, potential risk to humans becomes a matter of exposure.

More information on the early events in the process in male rats that could better delineate the relationship between melamine administration, urinary concentrations, and the formation of calculi and ulceration of the epithelium would be useful. Details regarding the differences between males and females and between rats and mice, including toxicokinetics, particularly regarding urinary melamine concentrations, could clarify some of the sex and species differences. Information on urinary concentrations in humans after occupational or environmental exposure could help clarify exposure issues.

In summary, although fundamental differences in biology make it clear that humans are less susceptible to the carcinogenic effects of calculi than rodents, calculi do appear to pose a small carcinogenic risk for humans. For a given chemical, risk is totally dependent on exposure.

IV. Statement of Confidence; Decision Analysis

Application of the human relevance framework to melamine carcinogenesis was useful overall. For data from laboratory animals, the well-established relationship between the formation of calculi and the ultimate development of bladder carcinomas satisfied the data expectations of the framework. It has become clear from these animal studies with melamine and other chemicals, as well as a few human studies with other chemicals, that this phenomenon occurs only at high doses, at doses at which the solubility of the substances forming calculi have been exceeded, leading to precipitation of either the test chemical or metabolite or an endogenous product formed because of alterations in the physiology of an animal (Clayson et al., 1995; IARC, 1999a, 1999b; Rodent Bladder Carcinogenesis Working Group, 1995). The level at which precipitation occurs can be greatly influenced by other urinary parameters such as osmolality, protein and citrate, among a variety of other substances depending upon specific cases (Cohen, 1995b). Once the precipitate begins to form, it can eventually grow to a size where a grossly visible calculus is formed that leads to cytotoxicity of the urothelium. In animal studies, the extent of the cytotoxicity depends on size, number, and coarseness of the calculi, and the length of time that they are present. This cytotoxicity frequently is sufficient to produce full-thickness damage to the epithelium, leading to ulceration and an inflammatory response. This leads to regeneration and hyperplasia, which ultimately can be repaired if the stimulus is removed, or leads to an elevated incidence of bladder epithelial tumors if the proliferation is prolonged. A similar toxicity and regeneration sequence secondary to calculi is observed in humans.

For melamine, although the data are limited, there is a strong correlation in rats and mice between calculi and bladder tumor formation. More detailed information from studies with other chemicals is available to bridge various gaps in data, such as urinary concentrations and a more thorough understanding of early events in the damage to the urothelium in response to melamine (U.S. EPA, 1984, 1988; Rodent Bladder Carcinogenesis Working Group, 1995; IARC, 1999a). However, since melamine is a non-DNA-reactive compound, no other plausible mechanism has been suggested, and because there is a strong correlation between formation of urinary calculi and a proliferative response in the urothelium, the evidence is sufficient for concluding that melamine produces bladder tumors in rodents by this mode of action.

There is significantly less risk in humans for developing bladder cancer from calculi than in rodents, most likely due primarily to the usually short time calculi are present in humans due to anatomic and obstructive issues (Burin et al., 1995; IARC, 1999b; Rodent Bladder Carcinogenesis Working Group, 1995). There is also the confounding factor of bacterial infection in the case of calculi in humans, making it impossible at this time to conclude whether it is the calculi alone or in combination with the infection that is producing the slight increased risk of development of bladder tumors.

Thus, there is a high degree of confidence in the mode of action of urinary-tract calculi producing bladder tumors in rodents. Based on epidemiologic investigations and similar biochemical, physiologic and pathologic processes in humans, there is a high degree of confidence that calculi are also associated with bladder tumors in humans. Humans appear less susceptible to the carcinogenic effects of calculi, and the association is a high-dose phenomenon only. Thus, exposure analysis becomes critical for a risk assessment of a given chemical acting by this mode of action. For melamine, there are no data available in humans so that the human relevance of the findings in animals is based entirely on extrapolation from results from other substances.

V. Implications

Urinary tract calculi produce an increased incidence of urinary bladder tumors in rodents by a non-DNA-reactive mode of action involving ulceration, regenerative hyperplasia, and formation of bladder tumors. For the chemicals studied to date, this is a high-dose phenomenon. The relationship between calculus formation and bladder tumor formation in rodents is strong. A wealth of evidence from investigations involving numerous chemicals makes clear the value of using information from several different sources to complete the analysis of human relevance.

The relationship of urinary tract calculi in general to bladder carcinogenesis in humans remains undefined, but possibly poses a small relative risk. This mode of action is applicable to humans. Humans appear to be less sensitive to the carcinogenic effect of calculi, and bacterial infection, a known risk factor for human bladder cancer, is a confounding variable. Since this mode of action is a high-dose phenomenon only, requiring sufficient exposure to produce precipitation in the urine, exposure analysis becomes critical to the proper assessment of cancer risk to a specific chemical.

Estimates of potential human exposure have been made. The pesticide cyromazine is fed to chickens, leading to metabolism to melamine in the body. Maximum levels of melamine have been estimated as 0.25 ppm in meat and eggs consumed by humans (Table 10) (U.S. EPA, 1984, 1988). The NOAEL for calculi and tumors in rats is 2250 ppm. Feeding cyromazine to layer hens at 5 ppm in the diet (highest rate) leads to a combined residue of cyromazine and melamine (in melamine equivalents) of a maximum of 0.25 ppm in meat and eggs. If the rats are estimated to consume 15 g of diet per day and weigh an average of 250 g, and if human ingestion of chicken is estimated at 44 g per day and eggs at 34 g per day in a 70-kg person, the margin of exposure for rats to humans is calculated to be 2.1×10^6 (U.S. EPA, 1984, 1988; NIOSH, 1998; National Library of Medicine [NLM], 1998; NTP, 1983). In addition, in several respects, humans appear to be less susceptible to the effects of calculi as related to bladder tumor development. Although specific toxicity data for melamine in humans are unavailable, it

TABLE 10
Melamine Exposures

Issue	Rat	Human
1. Exposure producing: not producing: tumors	4500 ppm (~300 mg/kg/day): 2250 ppm (~150 mg/kg/day) (20)	
2. Occupational exposure		About 43,000 workers potentially exposed to melamine during production and manufacture of formaldehyde resins (NIOSH, 1998)
3. Environmental exposure		Release in the United States of 82,000 kg to air, 240,000 kg to water, and 13,500 kg to land; exposure judged to be "low" (U.S. EPA, 1988; NLM, 1998)
4. Melamine potential exposure from use of cyromazine—chicken and eggs		2.1-Million-fold below the NOAEL in rats (U.S. EPA, 1984)

appears that human exposures are inadequate to produce urinary-tract calculi, and therefore, melamine is not expected to pose a carcinogenic risk for humans.

The evidence that the chemical in this case study, melamine, produces bladder cancer in rats by the calculus mode of action is convincing. Thus, if adequate exposures to melamine occur, this chemical could pose a cancer risk in humans. This question can be answered definitively only with a full exposure and risk assessment. However, although there is little information on melamine effects and exposure in humans, current and projected exposures are estimated at five to six orders of magnitude less than the observed effect level in rats. For this reason, melamine is not expected to pose a cancer risk to humans through the calculus MOA with present usages.

In summary, the final step in a risk assessment involving animal mode of action requires consideration of where the chemical falls on the gradient of high to low expected human exposure. In the case of melamine, the expected exposure is very low. Other chemicals with a comparable mode of action and concordance analysis might have a different result if the expected exposure is higher than the extremely low exposure expected for melamine.

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APPENDIX

This appendix provides abbreviated definitions for several concepts and terms used throughout this report. Figure A-1 is adapted from the 1983 NRC report.

Risk Assessment

The risk assessment/risk management paradigm (NRC, 1983, l994) describes risk assessment in terms of four distinct analyses with the

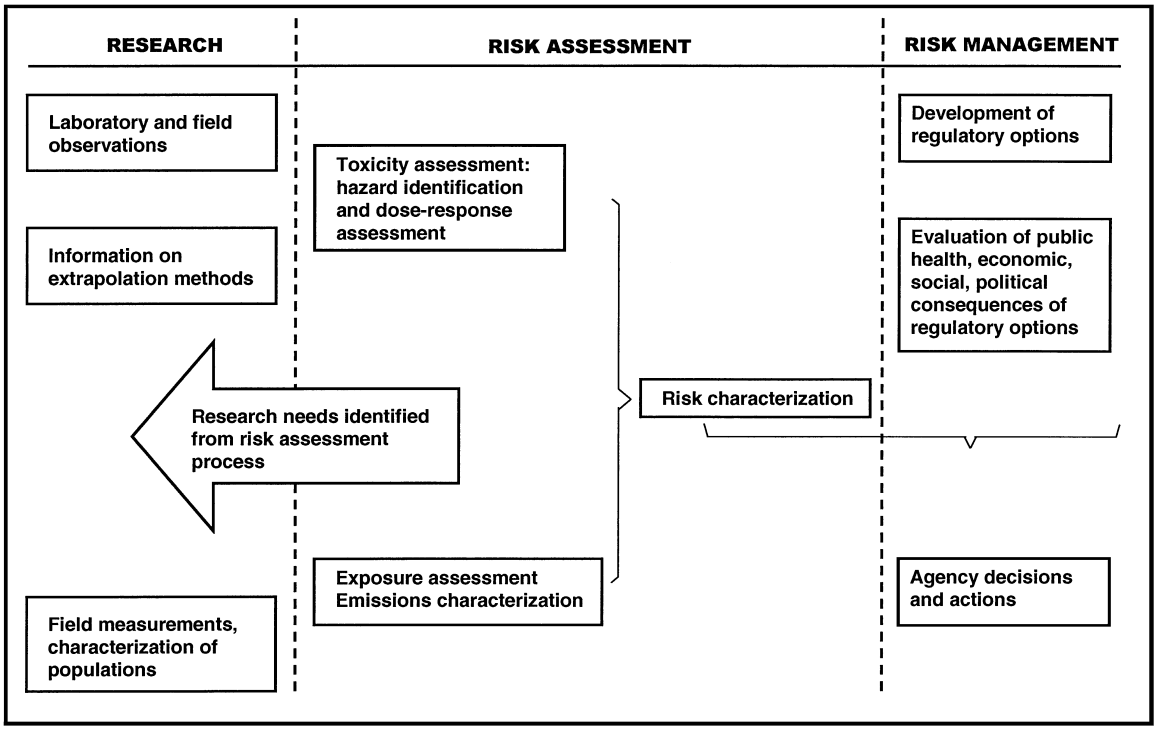

FIGURE A – 1 NRC risk assessment/management paradigm. Source: NRC (1994).

"risk characterization" end product destined for used in decision making. A diagram appears in Figure A-1, and relevant definitions follow.

Hazard identification. The process of determining whether exposure to an agent can cause an increase in the incidence of a health condition such as cancer or birth defects. It involves describing the nature and weight of the evidence of causation. Many practitioners and guidance documents refer also to *hazard characterization*, the presentation of the weight of evidence on hazard and, if MOA data are available, the MOA for an endpoint (or spectrum of endpoints) for defining appropriate endpoints for and approaches to dose/concentration-response assessment.

Dose/concentration-response assessment. The characterization of the relationship between the dose of an agent administered or received and the likelihood of an adverse effect.

Exposure assessment. The qualitative and/or quantitative assessment of the nature, form, and concentration of a chemical to which an identified population is exposed from all sources (air, water, soil, skin, and diet).

Risk characterization. The synthesis of data from exposure assessment, hazard identification and dose-response assessment into a summary that identifies clearly the strengths and weaknesses of the database, the criteria applied to the evaluation and validation of all aspects of methodology, uncertainties and data gaps, and conclusions reached, both qualitative and quantitative, including numerical risk values.

Toxicokinetics. How the body processes chemicals, including modeling and mathematical description of the interaction occurring at the interface of xenobiotics and tissues, as to such variables as similarities and differences in absorption, deposition, metabolism, elimination, and related parameters.

Toxicodynamics. How the body responds to chemicals, including differential sensitivity, modeling, and mathematical description of the time course of disposition of xenobiotics in the whole organism.

Weight of evidence. A systematic evaluation of factors bearing on the quality of all studies and other information, positive and negative, in the database under study.